JOURNAL OF THE NATIONAL CANCER INSTITUTE

National Institutes of Health • Public Health Service

Volume 27 • Number 5

November 1961

Immunohistochemical Studies on the Antigenic Relationship of Some Muscle Tumors to Normal Skeletal Muscle Cells [1, 2]

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SUMMARY

Normal skeletal muscle cells, when grown in tissue culture, appeared to shift from the production of myosin to a production of connective-tissue components. That an actual shift of production was taking place and that the observed change in cell population was not entirely due to an outgrowth of one particular type of cell with a decrease growth of another were shown clearly by a paired fluorescent-label technique. Individual cells could be found that contained both connective-tissue components and myosin. This change is of significance in connection with rhabdomyosarcomas in which a similar change would explain the previous observations that some human rhabdomyosarcomas contain myosin while others do not. The latter seem to contain connective-tissue components that are not observed in the rhabdomyosarcomas containing myosin. It would appear that the rhabdomyosarcomas not containing myosin were either not derived from muscle or had lost the ability to produce enough myosin to be seen and were producing enough connective-tissue components to be readily demonstrated.—J. Nat. Cancer Inst. 27: 937-947, 1961.

IN IMMUNOHISTOCHEMICAL studies with human rhabdomyosarcomas three cases of typical pleomorphic rhabdomyosarcomas did not contain enough myosin, if any, to give a positive test with antimyosin serum. Two of these tumors and part of a tumor from another individual stained with connective-tissue reactive antiserum, which indicated the presence of these antigens in the cytoplasm of the cells. Since the connective-tissue antigens did not appear to be in normal muscle cells, this raises a question of whether the tumors actually arose from muscle. If they did, they must have lost the ability to synthesize myosin and gained the ability to synthesize connective-tissue substances (*1*).

Studies were made in tissue culture of normal adult and fetal skeletal muscle and two embryonal rhabdomyosarcomas, in which myosin could be detected in the original neoplastic tissue, to see whether cells containing myosin could be followed in tissue culture and whether any antigenic alterations would occur.

[1] Received for publication April 3, 1961.
[2] Presented at the meeting of the American Association for Cancer Research, Atlantic City, N.J., April, 1961.
[3] The authors thank Mr. Joseph Bernecky and Dr. J. Jurandowski for technical assistance.

MATERIALS AND METHODS

The rabbit antimyosin antiserum (*1*) and the connective-tissue (C.T.) reactive antiserum (prepared against a human melanoma) (*2*) were previously described. The connective-tissue reactive nature of the latter antiserum was established by its reaction with various human organ sections. The antiserum reacts with the basement membrane of human kidney tubule as well as glomeruli, but not with the tubular epithelium. It reacts with the central veins and sinusoids of liver, but not with the liver parenchyma. The antiserum stains skeletal muscle in the endomysium. Absorption of the antiserum with human liver sediments removes all staining for the C.T. sites mentioned (*1*, *2*). The antiserum does not appear to react with parenchymal elements, but with C.T. The tissues that are used for absorption remove the capacity of the serum to react with reticular C.T. elements in all organs of human origin. We refer to this preparation as a "connective-tissue reactive antiserum."

In this study both the direct and indirect staining techniques were employed. In the direct procedure the globulin fraction of antimyosin serum was labeled either with fluorescein or tetramethylrhodamine isothiocyanate (*3*, *4*). This was also done for the connective-tissue reactive serum. Both conjugates of contrasting labels were then used on the fixed-muscle cell cultures. Each antiserum was applied to the sections separately to avoid dilution and incubated 1 hour. Washing between incubations was for 10 minutes. The order of the staining was also reversed in every case to see if comparable results would be obtained.

The procedure for the indirect staining has been described in another communication (*5*).

All antiserums used were absorbed with rat liver sediments to remove fluorescent materials that stained tissue nonspecifically and, in some cases, with human liver sediment to remove cross-reacting antibodies, *i.e.*, connective-tissue reactive antibodies (*6*).

Inhibition of staining.—The inhibition studies were carried out on cultured cells of muscle to demonstrate the specificity of the reaction. The cultures contained both muscle cells and outgrowths of fibroblasts. Acetone-fixed cells were treated for 2 hours with the appropriate unlabeled serum for inhibition, washed 10 minutes, and then treated with the fluorescein-labeled antibody for 1 hour.

Tissue cultures.—Explants of fetal human skeletal muscle and normal adult skeletal muscle were made on coverslips in Eagle's medium containing 10 percent calf serum. After 2 weeks of incubation, with feeding made twice a week, cultures showing good growth were taken for study. Each coverslip was removed and washed in saline to remove excess media, fixed in cold acetone for 10 minutes, washed in distilled water, and rinsed in saline before treatment with appropriate antiserum.

Other cell cultures growing in Eagle's medium and fetal calf serum were used as controls. These were obtained from Dr. George E. Moore and were established lines made from explants of a cancer of the ovary (F),

a malignant mesothelioma (B), and a cancer of the gallbladder (S), all from human source. The C3HIA and the L lines of cultured mouse cells, as well as the hamster Gale melanoma line, were used as controls.

Two explants of human embryonal rhabdomyosarcoma were made in the same manner as described for the other tissues. These were obtained from necropsy under sterile conditions and part was frozen immediately for comparison with cultured cells.

RESULTS

By the indirect staining procedure human muscle cells in culture containing myosin could be detected readily (fig. 1). Even cells which could not be identified morphologically were easily identified as muscle cells by the presence of myosin. Only a very small fraction of the cells in culture contained myosin and therefore only a small fraction was probably muscle cells.

When the indirect technique was used with the connective-tissue reactive antiserum, the extensive staining of the culture made it impossible to identify muscle cells with certainty. As may be seen in figure 3, muscle cells containing myosin and fibroblasts overlap one another and associate intimately so that it was not possible to ascertain whether muscle cells were actually stained or merely reflecting the fluorescence from the fibroblasts. As can be seen in figure 2, the fibroblast-appearing cells stain quite strongly in the region of cytoplasm; the nuclei were not stained.

Inhibition studies with the direct staining technique demonstrated specificity of the staining and showed that each antiserum was staining specifically and acting independently of the other against its homologous antigenic sites. Thus antimyosin antiserum prevented staining of muscle cells by fluorescein-labeled antimyosin, but did not interfere in any staining of cells by labeled connective-tissue reactive antibody. Connective-tissue reactive antiserum interfered completely with the staining of fibroblasts by the fluorescein-labeled preparation, but did not interfere in the staining of cells by fluorescein-labeled antimyosin antibody.

To determine whether muscle cells were staining with the connective-tissue reactive antiserum, we used a double-label procedure with tetramethylrhodamine on the connective-tissue reactive serum and fluorescein on the antimyosin serum. It was then possible to see myosin-containing cells growing intimately with the surrounding fibroblasts (fig. 3). With the fluorescein-labeled antimyosin serum as a reagent to locate myosin-containing cells free from surrounding stromal cells, observations could be made with the rhodamine-labeled C.T. reactive antibody, which showed that there were connective-tissue elements in the perinuclear regions of the muscle cells (fig. 4). Similar results were obtained when the labels on the two serums were reversed.

The results ran contrary to those seen for adult skeletal or fetal muscle sections in that the C.T. reactive antiserum did not appear to stain the

muscle elements in the section, but staining was confined to what appeared as endomysium (*1*). In tissue culture we can detect in the developing muscle cells the presence of stromal antigens as well as myosin.

Staining with the connective-tissue reactive serum was rather general for several tissue-cultured cell lines not previously tested with this serum. These tissue-cultured cells were obtained from a cancer of the gallbladder, a m alignant mesothelioma, and a cancer of the ovary. That staining was due to C.T. reactive antibody was shown by the fact that, if this antiserum was absorbed with human liver sediments, staining no longer occurred on any of these cells (*2*, *6*). Antimyosin serum absorbed with human liver sediments, although capable of staining some cultured muscle cells, *i.e.*, those containing myosin, did not stain any of the lines.

Cells of different species, *i.e.*, mouse lines C3HIA and L, and a hamster line, Gale melanoma A, did not stain with either antiserum.

Embryonal rhabdomyosarcoma cells taken from a 14-year-old child were cultured and gave the following results: Originally this tumor in frozen sections showed strong positive staining with antimyosin antibody, but not with connective-tissue reactive antibody. When allowed to grow for 4 weeks only, fibroblast-appearing cells were seen, and the rhabdomyosarcoma cells, which histologically appeared roundish and carcinomatous, were no longer visible in the culture. Treatment of the slides with antimyosin serum showed no convincing staining for the presence of myosin in any of the cells. Treatment with C.T. reactive serum stained all cells.

Another rhabdomyosarcoma, which contained fibroblastic as well as round cells in the solid tumor and stained strongly with only the antimyosin serum, was cultured (fig. 5). Cultures, 2 weeks old, started from the original solid tumor, were completely negative with antimyosin serum, but strongly positive with C.T. reactive serum. A subculture of this 2 weeks' growth was also tested after another 2 weeks of growth, but this again showed only sheets of fibroblast-like cells which did not stain with antimyosin serum (fig. 6).

Fetal muscle tissue (estimated age of fetus, 4–5 months) was cut in serial sections and stained with both antimyosin and C.T. reactive antiserum to determine whether any mesenchymal or muscle-appearing cells would show the presence of both myosin and connective-tissue antigens. In the limited number of fetuses studied, we have not been able to see any groups of cells containing both antigens.

DISCUSSION

Skeletal muscle has been studied extensively by many investigators. Godman (*7*, *8*) has reported on the cell transformation and redifferentiation of regenerating striated muscle and has shown that the events of regeneration are similar both *in vitro* and *in vivo*. Holtzer *et al.* (*9–12*) have studied the myogenesis of chick-embryo muscle. They have been

able to correlate the sequence of morphological differentiation with the appearance of intracellular myosin and with the capacity of the myoblasts to contract.

The explantation of an organ tissue, such as muscle, liver, or kidney, into tissue culture imposes certain conditions, *i.e.*, the explant no longer has the environment it did in the original state. Consequently, specific functions may become minimal or lost and other capacities may manifest themselves in the cell; these changes may be reflected as changes in physiological and biochemical activities of the cell (*13*, *14*). Since the enzyme constitution determines the activity of a cell, the changes reflect shifts in enzyme patterns, and, as a result, shifts in antigen concentration of various components. All propagating cell lines must of necessity adapt to the limited static environment imposed by tissue culture; such conditions may perhaps act as medium to select cells with common capacities, *i.e.*, cells that have "reverted" to utilizing common enzyme patterns reflecting production of the "common antigens." Therefore, continuously propagated mammalian cells are remarkably similar, though of different origin, and appear devoid of the specific functions and properties manifest by the tissue from which they were derived (*2*, *15*, *16*). An apparent exception is the culture of malignant mast cells by Schindler *et al.* (*17*) which continues to produce histamine and serotonin.

A distinction must be made between rapidly growing cells in culture and those recognized as "maintained" or "organ" cultures in which cell division appears not to be evident or occurring only at a very slow rate. The degree of adaptation in the latter must certainly not be as great, and certain specific function and identity are probably still maintained in culture (*18*).

The demonstration of connective-tissue antigens in the cultured muscle cells can be ascribed to one of two processes: (*a*) The muscle cells may actually have the capacity to synthesize these antigens, but they were present in undetectable amounts before culture, or (*b*) the muscle cells may be ingesting connective-tissue antigen from the culture fluid by pinocytosis while the fibroblasts are releasing these into the medium. Pinocytosis is more common in cells in culture than it is in the intact animal. In preliminary studies we were able to demonstrate that the C.T. reactive antiserum was capable of lysing viable muscle cells (containing myosin) in the presence of complement, which indicated that these cells do possess some substances capable of reacting with the C.T. reactive antiserum. Since the lytic system is more sensitive than the fluorescent immunohistochemical technique, one cannot assume that the "C.T. reactive antibodies" are the sole participants in this reaction, but the possible association of the antigen with the muscle cell is indicated.

Evidence that the first process is responsible may be cited. The connective-tissue reactive antiserum has the capacity to stain or lyse tissue-cultured fetal liver cells and many other cultured human cells from presumably different origin (HeLa, Raos, cancer of the ovary, malignant mesothelioma, and cancer of the gallbladder) and yet not react with the

adult liver parenchyma or other homologous adult cells (*2*, *15*). The antiserum, when properly absorbed with human tissue sediments to remove connective-tissue reactivity, no longer stained or lysed any of the cultured cells mentioned.

These observations indicate that all propagating cell lines must adapt to the environment imposed by tissue culture and as a consequence reflect the production of "common antigens." It may be that many or all cells of a species have the potential to produce these substances. If this is true, then many tissue-cultured cells from different organs, whether or not of connective-tissue origin, may revert to synthesizing these "common antigens" which have been inadequately described here as "connective-tissue antigens."

The demonstration of connective-tissue antigens in the cultured muscle cells means that there is a stage in their development when these cells have the capacity to synthesize both myosin and C.T. antigens. The original skeletal muscle therefore has the potential to produce both substances, and the fact that the fluorescent-antibody technique shows myosin content, but not the presence of stromal antigen, except in what appears to be the endomysium, may be due to limitations of the technique. These observations point to the possibility that the muscle cells can produce myosin or connective-tissue antigens or both. Perhaps during the developmental sequence from mesenchymal origin they acquire the ability to synthesize myosin and as the cells differentiate into adult muscle the myosin content increases with gradual diminution of the connective-tissue antigen. Perhaps the antigens are not completely lost from the cells, but are not produced in high enough concentration to be detected. This possibility is evidenced by the fact that in tissue culture the connective-tissue antigen again becomes detectable.

The observed rapid outgrowth of cells from a tissue explant in culture is usually attributed to the outgrowth of fibroblasts. Our observation that the outgrowth stains with connective-tissue reactive antibody certainly does not mean that these are organ cells which have rapidly reverted to producing connective-tissue antigens. In muscle, we know that myosin-containing cells do grow out slowly and the amount of connective-tissue antigen present is not comparable to that seen in the fibroblasts.

These results indicate that adult skeletal muscle may contain low quantities of "connective-tissue antigens," and a potential muscle tumor cell undergoing the transformation to neoplasm may either synthesize myosin as in embryonal rhabdomyosarcoma, or connective-tissue antigens as in pleomorphic rhabdomyosarcoma, with the exclusion of one or the other antigens. It may be possible that individual tumor cells may be found with both types of antigen present, as seen in the cultured normal muscle cells. Possibly cells may be present which contain no antigen of either group (*5*).

The many possible changes a malignant cell may undergo may make it difficult to establish a pattern by which to determine the cell type of origin. However, it does seem possible to determine the tissue of origin

when that tissue contains a unique type of substance found also in the tumor, *i.e.*, myosin in the rhabdomyosarcoma.

The observation that the ability to produce myosin is lost in some human rhabdomyosarcomas is similar to the observations made by Weiler in the rat (*19–21*). He found that, during carcinogenesis by 4-dimethylaminoazobenzene, certain components of normal liver were no longer detectable in the hepatoma, as shown by loss of fluorescent-staining activity with antinormal liver microsome serum. From these studies he visualized a shift of the antigenic spectrum of the tumor from normal liver, *i.e.*, the organ-specific antigen is lost during carcinogenesis and other antigens appear which are also found in a number of normal organs. He also found that certain components of hamster kidney tubule cells are lost when these cells are cultured.

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PLATE 134

FIGURE 1.—Muscle-culture cells treated with rabbit antihuman myosin serum absorbed with human liver sediments and subsequently stained with fluorescein-labeled horse antirabbit reagent. Staining is confined to those cells containing muscle myosin. Fibroblasts remain unstained in the section.

FIGURE 2.—Muscle-culture cells treated with rabbit antiserum reactive with human connective tissue (C.T.) absorbed with rat liver and subsequently stained with fluorescein-labeled horse antirabbit reagent showed general staining of the entire population of cells. One can see a large overgrowth of fibroblasts in the muscle culture. When fibroblasts are lying in close contact with muscle cells it is difficult to distinguish whether muscle cells were actually fluorescing or if the apparent fluorescence was due to reflection from the fibroblasts. If the C.T. reactive serum was absorbed with human liver sediments, no staining occurred on any cell.

FIGURE 3.—Muscle-culture cells treated with fluorescein-labeled rabbit antimyosin globulin absorbed with human liver sediments and subsequently treated with rhodamine-labeled C.T. reactive globulin absorbed with rat liver sediments.

There are two types of cells in the field. The green fluorescent cell has been identified as a muscle cell because it reacts with the fluorescein-labeled antimyosin serum. Other cells staining orange are identified as fibroblasts since they did not react with antimyosin serum but only with the rhodamine-labeled C.T. reactive antiserum. Although it has the morphology of a fibroblast, the myosin-containing cell in addition to staining green also shows the granular, orange fluorescence perinuclearly in the cytoplasm. As can be seen, the muscle cell grows intimately with the surrounding fibroblasts, so that it would be difficult to demonstrate and interpret the presence of C.T. antigen in the cytoplasm of the cell if a single labeled antibody is employed as in figure 2.

FIGURE 4.—Muscle culture treated as above. An isolated cell was located showing clearly the presence of the C.T. antigen situated perinuclearly.

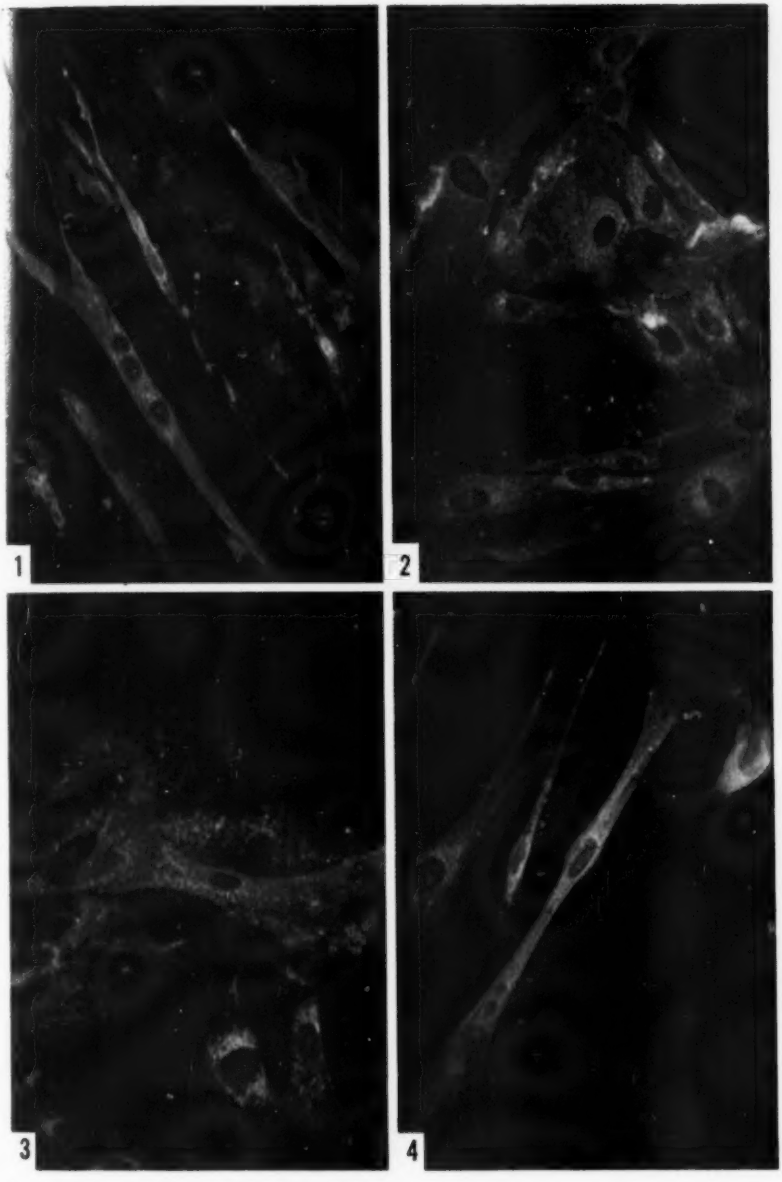

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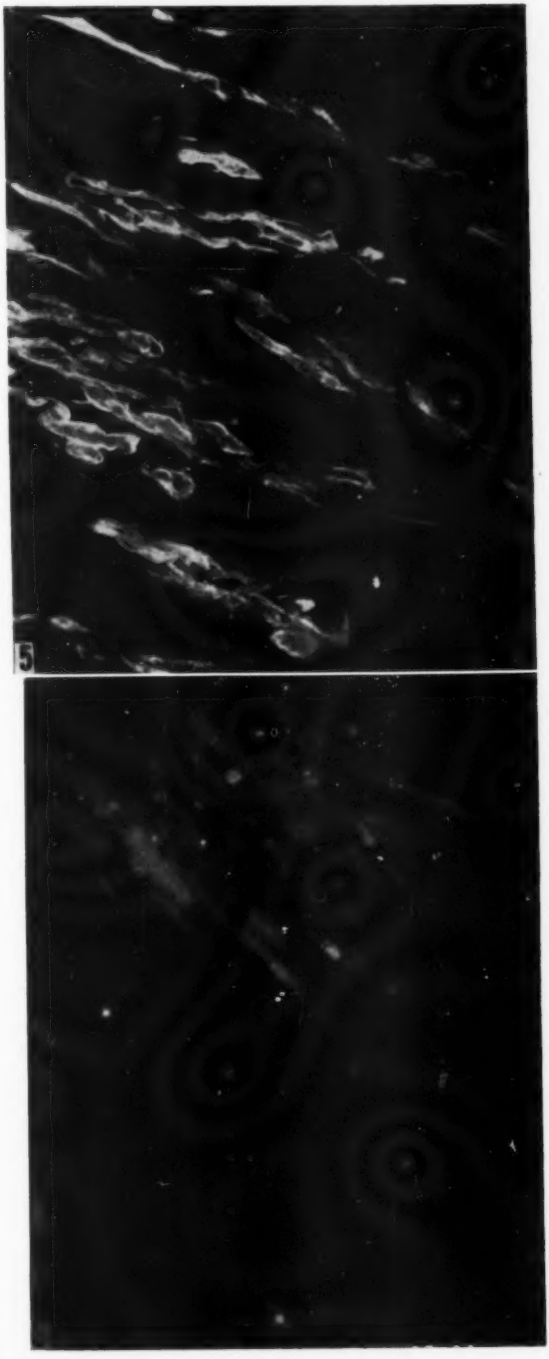
PLATE 135

FIGURE 5.—Embryonal rhabdomyosarcoma section treated with antimyosin serum and stained as described in text. The spindle-shaped rhabdomyosarcoma cells were stained intensely.

FIGURE 6.—Embryonal rhabdomyosarcoma tissue culture treated as above. Negative slide—no staining was seen with antimyosin serum, which suggests that none of the cells originally containing myosin was present. Cells showed only a weak blue autofluorescence and arrangement of fibroblasts can be barely seen in photograph. These cells stained strongly with connective-tissue reactive serum.



A Study of Mitotic Rate in Renewal Zones of Nondiseased Portions of Gastric Mucosa in Cases of Peptic Ulcer and Gastric Cancer, With Observations on Differentiation and So-Called "Intestinalization" of Gastric Mucosa [1, 2]

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SUMMARY

The mitotic rate in the zone of renewal was determined in the body and pyloric mucosa and in areas of so-called intestinalization outside the actual disease process in human stomachs removed, partially or totally, for duodenal ulcer (32 cases), gastric ulcer (35 cases), and gastric cancer (17 cases). In the material from stomachs of gastric-ulcer and cancer patients the mitotic rate in the body and pyloric mucosa was of the same magnitude and was highly significant ($P<0.001$) since it doubled that of corresponding sites in material from duodenal-ulcer patients. In all the material, the mitotic rate in the pylorus was significantly higher ($P<0.01$) than that of the body. Also, the mitotic rate in intestinal-type glands in material from gastric-ulcer and cancer patients was slightly greater than in materials from duodenal-ulcer patients. Intestinal-type glands in the pyloric region were observed in 78 percent of the cancer, 60 percent of the gastric-ulcer, and 25 percent of the duodenal-ulcer groups. They were more numerous in the pyloric part than in the body mucosa, and considerably more profuse in the first 2 groups. Intestinalization of the gastric mucosa has been considered primarily a sign of general differentiation disturbance. Gastric-ulcer and cancer stomachs showed, in addition to intestinalization in the superficial epithelium, incomplete cell differentiation and numerous pyknotic and karyorrhectic cells. These changes were rare in duodenal-ulcer cases. The highest incidence of cell renewal, of intestinal-type ephithelium and other differentiation disturbances, and of stomach cancer occurred in the same age group and may be related phenomena.—J. Nat. Cancer Inst. 27: 949-971, 1961.

THE MITOTIC activity of human organs has not been studied as extensively as that of animals because it is difficult to obtain fresh material from living man. With surgically removed tissues some knowledge of the renewal of cells can be attained, a process which must otherwise be envisaged by inference from studies of test animals. Of course, material of this character cannot be compared directly, *e.g.*, with biopsy material obtained at gastroscopy since the stress affects the mitoses (*1*). It is,

[1] Received for publication March 7, 1961.

[2] This study was supported by grant DRG-291-E-F from the Damon Runyon Memorial Fund for Cancer Research and by the Sigrid Jusélius Foundation.

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however, possible to compare different disease groups from human surgical material if specimens are obtained under similar conditions.

The epithelium of the alimentary canal is one of the labile organs and undergoes throughout life a continuous renewal of cells. Diurnal renewal of 35.4 percent of the surface epithelial cells occurs in the rat stomach and of 15.6 percent of mucous neck cells in the body part of the stomach; in the pyloric part the corresponding renewal rate is 51.6 and 56.4 percent, respectively (*2, 3*). Renewal is more rapid in the intestinal mucosa, 70.4 percent daily (*4*).

The mitotic rate is fairly constant under normal conditions and hence it is possible to speak of the mitotic homeostasis of the organ. It can be affected for a short time, *e.g.*, by radiation (*5, 6*), hormones (*7*), and karyoclastic agents (*8*), but this influence returns to the original level. Mitotic activity also shows physiological variation, *e.g.*, diurnal rhythm (*9*).

Regeneration of glands of the body mucosa and pyloric mucosa of the stomach takes place in the neck part, with the undifferentiated cells dividing mitotically (*10*); concurrently with their differentiation they penetrate deeper into the mucosa or move to the surface where they make up the surface epithelium. Normally, no mitoses occur in the parietal and chief cells of the gastric mucosa of adult man or in the cells of the superficial epithelium.

Intestinal-type epithelium is said not to occur in the mucosa of normal human stomach (*11*). But argentaffin cells, which are actually cells of the intestinal mucosa, occur normally in the pyloric part of the human stomach.

Numerous mitoses have been observed in the mucosa of the human stomach in the hyperplastic regions of the surface epithelium in cases of peptic ulcer and cancer (*12–14*) and in test animals when carcinogens influence the gastric mucosa (*15*). Mitotic figures have also been demonstrated in the immediate vicinity of experimental ulcers (*16*).

The presence of intestinal mucosal cells in the stomach of patients with pernicious anemia has long been known as a typical feature of this disease. Intestinalization of the stomach, on the other hand, occurs profusely in gastric ulcer and cancer patients (*12, 13, 17–19*), and to a less extent in duodenal-ulcer patients (*12, 18, 19*). Intestinal-type cells have been found in great numbers in gastric cancers (*20*).

Intestinal glands in the gastric mucosa have been most numerous in the vicinity of an ulcer (*19*), and it has been suggested that ulcer is cured by intestinal mucosa (*13*). It has been observed that mucosal lesions in the stomach of test animals heal with the help of intestinal-type mucosa (*21, 22*) or of gastric mucosa of normal structure (*16, 23, 24*). Regeneration of normal mucosa, which covers the ulcer, has also been reported in man as part of the process of healing gastric ulcer (*23*).

Gastric cancer occurs more frequently in the stomachs of patients with gastric ulcer (*25–28*) than in the population as a whole or in duodenal-ulcer patients. In addition to intestinalization, the gastric mucosa of gastric-ulcer and cancer patients often displays hyperplastic changes in

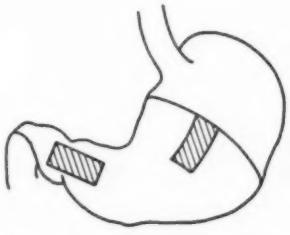
cells of the superficial epithelium. These changes may indicate a tendency to malignant transformation (*13, 17, 18, 28, 29*).

MATERIAL AND METHOD

The material consisted of unselected stomachs obtained at routine operations and is listed in table 1. The distribution of the disease group was as follows:

	Number of cases		
	Males	Females	Age ranges
Duodenal ulcer	30	2	30–65
Gastric ulcer	23	12	19–73
Gastric cancer	12	5	32–76

The mucosal samples were taken in connection with an operation on the stomach. In partial or subtotal gastrectomy the sample was a strip 3 to 5 cm long and 1 to 1½ cm wide taken from the oral and pyloric end of the resected part immediately after removal of the stomach. The samples taken were outside the actual pathological process. Samples were also obtained from the ulcer and the cancer for examination of the mitotic activity of the mucosa in their immediate proximity. They were cut longitudinally to the stomach, from the anterior wall, and equidistant from the greater and the lesser curvature (text-fig. 1). Before immersion in 10 percent formalin solution for 2 to 3 days, the mucosal strips were smoothed out on cardboard.



TEXT-FIGURE 1.—Sites of specimens taken from the anterior wall of the stomach.

The formalin-fixed samples were cut perpendicularly to the surface of the mucosa. Paraffin sections were cut 4 μ thick and stained with hemalum and eosin. A magnification of 1350 and oil immersion were used.

Mitotic count in the ordinary gastric epithelium.—The mitotic count was made in the mitotic area of the glandular tubules. In the body mucosa, the mitoses were counted in the vicinity of the isthmus, limited in depth to the regular parietal-cell zone and in the direction of the surface to the fundus of the foveola where the cells of the superficial epithelium of the pits began (text-fig. 2). Correspondingly, in the pyloric mucosa the counting was terminated at the typical cells with disc-shaped nuclei of

TABLE 1.—Mitotic activity and intestinal-type epithelium in the whole material

Case No. and initials	Age and sex	Body mucosa: Normal gastric glands: Mitoses (percent)	Body mucosa: Intestinal-type glands: Degree of appearance	Body mucosa: Intestinal-type glands: Mitoses (percent)	Body mucosa: Intestinal-type glands: Argentaffin cells (percent)	Pyloric mucosa: Normal gastric glands: Mitoses (percent)	Pyloric mucosa: Intestinal-type glands: Degree of appearance	Pyloric mucosa: Intestinal-type glands: Mitoses (percent)	Pyloric mucosa: Intestinal-type glands: Argentaffin cells (percent)	Local pathological process: Intestinal-type glands: Degree of appearance	Local pathological process: Intestinal-type glands: Mitoses (percent)	Local pathological process: Intestinal-type glands: Argentaffin cells (percent)	Duodenum: Intestinal glands: Mitoses (percent)	Duodenum: Intestinal glands: Argentaffin cells (percent)
DUODENAL ULCER														
1) MK	30♂	4.7	0			3.9	0							
2) RV	32♂	5.7	0			4.0	0							
3) VH	32♂	2.7	0			3.6	0							
4) RP	32♂	2.3	0			3.4	0							
5) HK	35♀	3.6	0			4.6	0							
6) TS	35♂	3.3	0			4.1	II	2.9	1.27					
7) TT	36♂	5.2	0			3.1	0							
8) OT	36♂	2.4	0			2.3	0							
9) VK	36♂	4.7	0			8.8	II	3.3	1.90					
10) JN	37♂	1.6	0			1.5	0							
11) EL	38♂	1.4	0			3.5	0							
12) LL	39♂	5.2	0			6.2	0							
13) AK	41♂	5.4	0			6.5	0							
14) YR*	42♂	2.8	0			1.8	II	2.3	1.4				1.8	1.2
15) VT	43♂	2.3	0			3.4	0							
16) TH	44♂	3.1	0			4.2	0							
17) AK	45♂	1.9	0			2.4	II	1.8	1.0				1.1	4.4
18) VP	45♂	2.3	0			2.3	0							
19) HL	45♂	2.9	0			6.0	0							
20) VR	47♂	1.4	0			3.0	I	1.3	0.7					
21) HB	48♂	5.9	0			6.6	0							
22) ML	48♂	2.6	0			4.9	0							
23) VJ	48♂	1.9	I	1.3	1.6	3.8	0						2.1	1.3
24) HS	49♀	4.3	0			5.8	0							
25) AA	49♂	2.7	0			3.9	0							
26) PK	50♂	1.4	0			1.3	I	2.5	†					
27) RH	51♂	9.2	0			9.0	III	5.8	†					
28) AK	52♂	1.4	0			‡								
29) OP	53♂	8.2	0			7.8	0							

30) LL	58♂	2.2	0			2.5	II	0.8	0.9					
31) VP	60♂	4.0	II	0.6	1.5	3.5	0							
32) OK	65♂	1.4	0			5.8	0							
GASTRIC ULCER														
33) LV	19♀	9.5	III	4.1	0.7	11.9	0			II	†			
34) JL	31♀	2.6	0			3.1	0						0.6	0.3
35) BS	35♂	8.0	II	3.9	1.1	5.9	III	4.1	0.4					
36) LK	37♀	10.7	II	3.7	1.4	8.9	II	4.2	1.3					
37) VK	40♂	9.8	II	5.4	0.2	9.0	0			II	4.0	2.1		
38) EA	44♂	1.7	0			5.7				0–I	2.0	0		
39) KK	44♂	6.5	0			9.1	I	3.4	0.8					
40) UK	44♂	10.2	0			13.3	I	5.2	†					
41) OJ	45♂	8.4	0			7.2	0			0				
42) IS	45♀	5.6	0			6.9	0			0				
43) IV	49♂	4.3	0			6.1	II	2.7	0.2					
44) HV	49♂	5.9	0			6.8	I	3.9	1.9	II	3.2	1.1		
45) LK	50♂	6.8	I	2.3	1.2	7.0	II	2.8	†	0				
46) HT	51♂	7.6	0			6.6	0							
47) EH	51♂	7.9	I	2.6	3.0	§	III	2.5	0.4	II	1.0	2.5		
48) AH	52♂	6.8	II	5.0	2.3	7.8	II	4.4	1.8					
49) JK	53♂	8.2	I	5.7	0	11.5	0			III	5.5	0.7		
50) AA	55♂	5.6	0			6.9	III	4.6	0.1					
51) HM	55♀	10.2	0			12.3	II	4.2	0.1	0				
52) EV	55♀	7.1	II	3.8	2.0	7.0	II	4.2	1.5	II	3.5	0.6	3.7	1.8
53) TA	56♂	4.0	0			§	III	5.4	0.3	II	4.3	0.7		
54) PK	56♂	3.6	0			4.4	I	2.5	0.5				1.9	2.4
55) VN	57♂	8.1	0			6.6	III	2.3	0.6	I	2.4	0.4		
56) AV	57♂	5.1	0			7.4	II	2.6	1.2					
57) SW	57♂	4.5	III	2.1	1.0	4.0	0							
58) LK	58♀	3.3	I	2.2	2.6	3.7	0–I	†					3.2	5.8
59) EP	59♂	6.9	0			§	III	3.3	0.7	I	1.8	3.2		
60) SM	60♂	4.7	I	2.2	1.6	4.1	0			I	†			
61) HK	60♀	6.3	0			8.8	0							
62) IR	61♀	5.9	0			6.5	0			0				
63) EL	63♂	7.6	II	2.0	3.4	8.0	I	3.5	0.2	I	3.5	0.4		
64) JH	66♂	3.1	II	1.3	1.0	5.5	II	2.3	2.6	0				
65) IH	67♀	5.5	0			5.0	II	3.4	1.6	0				
66) VU	70♀	5.0	0			7.5	II	2.5	0.3					
67) AT	73♀	4.7	0			7.4	0							

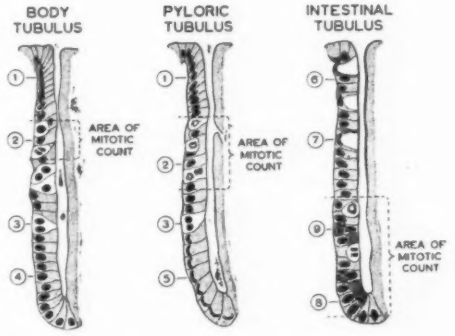
TABLE 1.—Mitotic activity and intestinal-type epithelium in the whole material—Continued

Case No. and initials	Age and sex	Body mucosa: Normal gastric glands: Mitoses (percent)	Body mucosa: Intestinal-type glands: Degree of appearance	Body mucosa: Intestinal-type glands: Mitoses (percent)	Body mucosa: Intestinal-type glands: Argentaffin cells (percent)	Pyloric mucosa: Normal gastric glands: Mitoses (percent)	Pyloric mucosa: Intestinal-type glands: Degree of appearance	Pyloric mucosa: Intestinal-type glands: Mitoses (percent)	Pyloric mucosa: Intestinal-type glands: Argentaffin cells (percent)	Local pathological process: Intestinal-type glands: Degree of appearance	Local pathological process: Intestinal-type glands: Mitoses (percent)	Local pathological process: Intestinal-type glands: Argentaffin cells (percent)	Duodenum: Intestinal glands: Mitoses (percent)	Duodenum: Intestinal glands: Argentaffin cells (percent)
GASTRIC CANCER														
68) HR	32♀	10.4	0			9.1	0			0				
69) KT	34♀	11.1	0			10.2	I	3.3	3.2	0				
70) EN	47♂	5.3	0			4.6	II	3.8	0.5	I	†			
71) IH	48♀	0.8‖				1.5	II	2.5	0.9	0‖		1.4		0.1
72) JA	48♂	0.8‖	0			¶								
73) RH	49♂	5.5	III	2.6	0.5	5.4	III	1.6	1.7	II	†			
74) MP	52♀	5.3	III	3.5	1.4	6.6	II	1.6	0.1					
75) AM	56♀	5.0	0			1.9	0							
76) EV	58♂	6.7	II	2.6	1.1	9.4	III	5.3	0.2					
77) VT	63♂	7.3	0			9.9	II	2.0	0.5	0				
78) VL	63♂	7.1	II	2.1	2.5	9.8	II	2.3	1.4					
79) JK	66♂	3.8	II	1.4	0.7	5.3	II	3.4	0	0				
80) HK	66♂	§	III	1.3	0.2	¶								
81) VT	66♂	5.6	II	3.6	1.6	9.4	0			I	1.7	0.4		
82) JT	68♂	3.8	0			5.8	I	2.5	0.7	II	1.5	1.1		
83) AM	72♂	1.8	0			2.5	II	2.5	0.1					
84) PH	76♂	3.4	0			¶								

*Perforating duodenal ulcer.
†Not counted.
‡Not taken.
§No normal glands.
‖Carcinomatously changed mucosa.
¶Not taken; gastrostomy.

the pyloric tubules, and advanced toward the surface when the cells of the pits entered the field of vision.

Mitotic count in the intestinal epithelium.—In ordinary intestinal tubules mitotic activity occurs in the fundal part of the glandular tubules. This was also true in heterotopic mucosa. The count in these glandular tubules began from the base of the crypt and ended in the layer in which, on advancing toward the surface of the mucosa, the striated border cells containing numerous goblet cells were seen. This area was mitotically inactive toward the oral part of the tubule and was omitted from the count (text-fig. 2). In samples from outside the pathological process it was generally possible to count the mitoses from fully developed intestinal tubules that displayed fully differentiated Paneth, argentaffin, striated border, and goblet cells. The edge of the ulcer or the cancer showed only tubules in the initial stage of intestinalization, which often originated from hyperchromatic nonspecific cells that could contain striated border and goblet cells. When there were no fully developed intestinal tubules in the samples, the mitoses were counted in the same way as from intestinal tubules.



TEXT-FIGURE 2.—The schematic drawings of the body, pyloric, and intestinal tubules in which the areas of mitotic count are noted. 1) Superficial epithelial cell, 2) mucous neck cell, 3) parietal cell, 4) chief cell, 5) pyloric cell, 6) striated border cell, 7) goblet cell, 8) Paneth cell, 9) argentaffin cell.

Mitoses were counted only in tubules that were sectioned longitudinally to the glandular tube. The parts cut tangentially were disregarded. All glandular cells and mitotic cells from prophase to anaphase at the margin of the tubule in question were included in the count. In each sample, 2,000 cells were counted from the given type of tubule and the results recorded as mitotic cells percent of the total cell count.

The degree of intestinalization was graded as follows:

Grade 0 = no intestinal-type glands.
Grade I = few single intestinal-type glands.
Grade II = numerous single or small groups of intestinal-type glands.
Grade III = numerous large groups of intestinal-type glands or complete intestinalization of the mucosa.

The Student's t test was used in comparing the mitotic activity of the different disease groups.

RESULTS

General Observations

The area extending from the fundus of the foveolae in depth to the parietal cells was mitotically active in normal-looking body mucosa. In stomachs from duodenal-ulcer patients, it was generally very shallow, about one fifth to one twelfth of the thickness of body mucosa as a whole. In stomachs from gastric-ulcer and cancer patients, outside the local pathological process, this area was generally considerably deeper, tortuous, and adenomatous; the fundal part of the tubule with its chief cells and parietal cells was correspondingly shortened. This lengthened neck part of the tubules sometimes constituted as much as half the over-all length of the glandular tube.

The mitotically active area of the pyloric tubules was thicker than in the body mucosa. Here again stomachs from gastric-ulcer and cancer patients showed more profuse hyperplasia of the neck part than those from the duodenal-ulcer patients.

No mitoses were established in the cells of the superficial epithelium in normal-looking body (fig. 1) and pyloric mucosa. Notable changes, however, were seen in the structure. The nuclei of the superficial epithelium were at different levels relative to the base of the cell in 2 stomachs from duodenal-ulcer patients, in 18 of those from gastric-ulcer, and in 8 of those from cancer patients. The cells were shallower and stratified one upon the other in the superficial epithelium, forming small papillomas in places (figs. 2 and 3). Degenerated superficial epithelium of this kind also showed marked pyknosis and karyorrhexis of the cell nuclei.

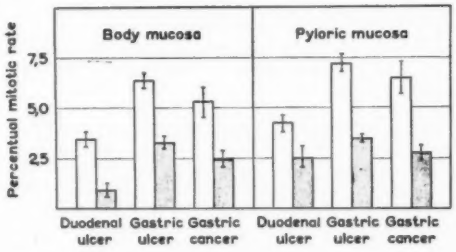
Mitotic Rate in Typical Gastric Mucosa

The results of the mitotic count are shown in table 2 and diagrammatically in text-figure 3.

The duodenal-ulcer patients showed less mitotic activity than the gastric-ulcer and gastric-cancer patients. This difference was highly significant ($P<0.001$) in relation to the gastric-ulcer patients and almost significant ($P<0.05$) in relation to those with gastric cancer. As hyperplasia of the isthmus caused the zone of regeneration to occupy a greater

Table 2.—Mitotic rate, percent ± standard error, in the original gastric glands

	Number	Body glands	Number	Pyloric glands
Duodenal ulcer	32	3.44 ± 0.35	31	4.28 ± 0.37
Gastric ulcer	35	6.33 ± 0.37	32	7.24 ± 0.42
Gastric cancer	16	5.23 ± 0.75	14	6.52 ± 0.84
Whole material	83	5.01 ± 0.28	77	5.92 ± 0.31



TEXT-FIGURE 3.—Percentual mitotic rate ± error in body and pyloric tubules (*unshaded columns*), and in the heterotopic intestinal tubules (*shaded columns*).

proportion of the gland length in both the body mucosa and the pyloric mucosa in stomachs of patients with gastric ulcer or cancer, the lesser renewal rate in duodenal ulcer was even more striking (fig. 4). In figure 5 an increased mitotic activity is seen in a tubule consisting of ordinary cells of the body mucosa in a case of gastric ulcer.

Normal-looking pyloric mucosa seemed to show a slight increase in mitotic activity compared with body mucosa in all the disease groups. In the material as a whole the mitotic rate of body mucosa was 5.01 ± 0.28 and of pyloric mucosa, 5.92 ± 0.31. The difference was statistically significant ($P<0.01$).

Frequency of Intestinalization

Intestinalization was established in the gastric mucosa in small islets containing a few glandular tubes or wide areas, especially in gastric-ulcer and cancer stomachs. In the small islets the glandular tubes were generally as long as the normal glandular tubes beside them. Their crypts showed a varying number of Paneth, argentaffin, and mitotic cells. Toward the surface, the goblet and striated border cells increased while the Paneth and argentaffin cells disappeared.

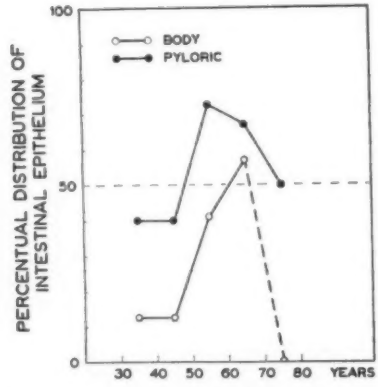
The areas of intestinal mucosa, which occurred in large islets, formed true villi in which there were no gastric cells of specific type. Such mucosa was thinner than normal gastric mucosa but thicker than the intestinal mucosa of the duodenum, seen in some samples.

In addition to fully differentiated intestinal tubules in the mucosa it was also possible to observe other kinds of tubules that had grown from the surface to various levels of the mucosa. Most of the cells in these tubules were similar in appearance to the mucous neck cells, except that their nuclei were deeply stained. They were possibly tubules in the initial phases of intestinalization (fig. 6).

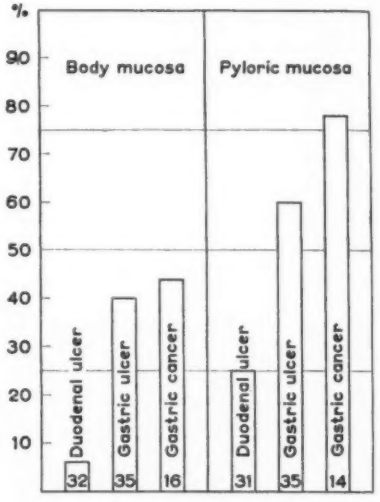
The incidence of intestinal heterotopia in the samples studied increased with age (table 3 and text-fig. 4). Intestinalization was more profuse in pyloric samples than in body mucosa. Text-figure 5 shows how intestinalization differed in the disease groups. It was scanty in stomachs from duodenal-ulcer patients, but considerably greater and of the same magnitude in those from gastric-ulcer and cancer patients.

TABLE 3.—Rate of the specimens with intestinal-type glands by age, in years, and by diseases

	19–30	31–40	41–50	51–60	61–70	71–80	Total
Duodenal ulcer:							
Body mucosa		0/12	1/14	1/5	0/1		2/32
Pyloric mucosa		2/12	4/14	2/4	0/1		8/31
Gastric ulcer:							
Body mucosa	1/1	3/4	1/8	7/16	2/5	0/1	14/35
Pyloric mucosa	0/1	2/4	5/8	11/16	4/5	0/1	22/35
Gastric cancer:							
Body mucosa		0/2	1/4	2/3	4/6	0/2	7/17
Pyloric mucosa		1/2	3/3	2/3	4/5	1/1	11/14



TEXT-FIGURE 4.—Age distribution of patients with intestinal epithelium in the whole material.



TEXT-FIGURE 5.—Percentual distribution of cases with intestinal mucosa in different disease groups.

The figures indicating the frequency of intestinalization are obviously too low because the investigation was confined to a relatively small part of the stomach. The real frequency is probably higher in the different disease groups, but the ratio between groups is probably the same as that given in text-figure 5.

Intestinalization occurred in large islets in the gastric-ulcer and cancer stomachs, but as sparse, small islets in the duodenal-ulcer stomachs. There were three gastric-ulcer cases in which the pyloric samples contained only intestinal mucosa.

Intestinalization occurred also in the proximity of the ulcer and in the cancer itself. In those gastric-ulcer samples that included an amount of the mucosa contiguous to the ulcer sufficient for evaluation, intestinalization was established in islets of varying size in 14 of 24 samples, *i.e.*, 58 percent. This corresponds to the frequency of intestinal mucosa

established elsewhere, *i.e.*, outside the local process in gastric-ulcer cases. It seems that the proximity of the ulcer does not affect the incidence of intestinal mucosa in gastric-ulcer stomachs.

Mitotic Rate in Intestinal Tubules

The mitotic activity of the glandular tubules was counted in 2 oral and 8 pyloric samples of the duodenal-ulcer material, in 14 oral and 21 pyloric samples of gastric-ulcer material, and in 7 oral and 11 pyloric samples of the gastric-cancer material. Their mitotic activity is shown in table 4 and text-figure 3. It will be observed that mitotic activity in the intestinal tubules is slightly less in the duodenal ulcer than in the gastric-ulcer and cancer samples.

TABLE 4.—Mitotic rate, percent ± standard error, in the intestinal-type glands in the body and pyloric gastric mucosa

	Number	Body mucosa	Number	Pyloric mucosa
Duodenal ulcer	2	0. 95 ± 0. 35	8	2. 58 ± 0. 54
Gastric ulcer	14	3. 30 ± 0. 27	21	3. 52 ± 0. 19
Gastric cancer	7	2. 44 ± 0. 34	11	2. 80 ± 0. 31
Whole material	23	2. 83 ± 0. 28	40	3. 14 ± 0. 18

Intestinal mucosa in the vicinity of the ulcer was often not contiguous to the regenerating mucosa at the margin of the ulcer but occurred in isolated islets there, too, as elsewhere outside the local process. The mitotic rate was 3.12 ± 0.42 percent in the vicinity of the ulcer and in intestinal islets about 2 to 4 mm from it. Mitotic activity was thus of the same magnitude as elsewhere in the intestinal islets occurring in gastric-ulcer stomachs.

Normal duodenal mucosa was studied in 6 samples. The mitotic index in this normotopic intestinal mucosa was 1.95 ± 0.15 in 2 duodenal ulcer cases, 2.9 ± 0.93 in 3 gastric-ulcer cases and 1.4 in 1 case of gastric cancer.

Argentaffin cells were also counted in the intestinal areas where they stained strongly. In these 15 samples, when the mitotic activity was high in the tubule (over 4%) the number of argentaffin cells was smaller (average 0.82%) than in the intestinal tubules where the mitotic activity was below 3 percent. Cell differentiation may be less developed in a more mitotically active area (figs. 6 and 7). A mitotic cell, which was eosinophilically granulated like an argentaffin cell, was observed in 1 case in a heterotopic intestinal tubule.

DISCUSSION

In normal-looking human body mucosa and pyloric mucosa, mitoses occurred in the mucous neck cells of the isthmus of the glandular tubes. No mitotic activity was established in the parietal, chief, or pyloric cells. It has been found, however, that these cells retain their mitotic potency in the regeneration of a traumatic lesion in rat gastric mucosa (*16*).

Superficial epithelium rarely displayed mitoses, contrary to the superficial epithelium of the mucosa of the rat stomach, which had a higher renewal rate than the mucous neck cells (*2*). The cells of the superficial epithelium showed pyknosis and karyorrhexis.

In gastric intestinal-type tubules and normotopic duodenal mucosa, the mitotically active area is the crypt. The specific cells of the crypt, argentaffin and Paneth, were more profuse the more completely the tubules penetrated the mucosa. The renewal rate was lively in the intestinal tubules that extended down into the mucosa for a short distance, but they contained few specific cells of the intestinal tubule. The oral part of the intestinal tubule consisted of goblet and striated border cells with scanty mitoses but numerous pyknotic and karyorrhectic cells.

In the duodenal ulcer the mitotic activity was clearly less in normal-looking body and pyloric tubules than in the corresponding gastric-ulcer and cancer patients. This difference was actually more pronounced than it seemed because the mitotically active part in the gastric-ulcer and cancer stomachs appeared longer and more hyperplastic than in those of the duodenal ulcer; apparently cell differentiation had been retarded in the former stomachs and with relative elongation of the isthmus. The fundal part of the tubules with its specific cells was correspondingly shorter in mucosa from ulcer or gastric-cancer patients. Many instances of comparable changes in gastric-ulcer and cancer stomachs have been reported in the literature and an exceptional number of mitoses have been observed in these conditions (*12–14*). Morphologically similar hyperplastic changes have been produced experimentally in the mucosa of animal stomachs (*15, 21, 30–33*).

The higher renewal rate in gastric-ulcer and cancer patients probably signifies a more rapid turnover of the gastric mucosa. If the mitotic time is the same, it is approximately double the turnover of the gastric mucosa of duodenal-ulcer patients. The turnover is somewhat more rapid in the pyloric than in the oral part of the stomach, as reported also for test animals (*2, 3*). No greater incidence of pyknosis, karyorrhexis, or karyolysis was observed in the specific cells of the fundal part of the glandular tubes, *viz.*, the chief cells and the parietal cells, though they did vary in number. The cells of the superficial epithelium, however, often showed signs of necrobiosis. Also, in gastric-ulcer and cancer stomachs hyperplasia and small signs of dysplasia of the superficial epithelium occurred. It is possible that these cells of the superficial epithelium reflect increased turnover and incomplete differentiation. Berg suggested that such hyperplasia and dysplasia reflected a generalized tendency to gastric neoplasia (*29*).

The frequency of intestinal metaplasia in the mucosal samples increased with the patients' age. This finding concurs with earlier observations (*18, 19*). With age, too, the gastric-ulcer and cancer patients constituted most of the material. There was a slight increase in mitotic activity with age; the maximum value was recorded in patients 50 to 60 years of age. This may occur because the nature of the disease changes with age.

Skin mitotic activity also increases with age (*34*). The occurrence in the same age group of intestinal metaplasia, mitotic activity, and cancer-frequency peaks is compatible with the possibility that these phenomena may be correlated.

In ulcer cases, the incidence of intestinal glandular tubes in the vicinity of the ulcer was not particularly higher than elsewhere. Possibly intestinal epithelium has nothing to do with the local ulcerous process. It is probably not necessary for regeneration of the ulcer, but a sign of a general differentiation disturbance of the stomach. [In fact, in experimentally induced ulcers regenerating nonspecific cells differentiate directly into normal gastric glands (*16*, *23*, *24*).] The mitotic rate of the intestinal glands in the vicinity of the ulcer was of the same magnitude as in the more distant intestinal glands.

In addition to fully differentiated intestinal glandular tubes there were, especially in the gastric-ulcer and cancer stomachs, glandular tubes that grew to different depths in the mucosa; the deeper cells of these tubes resembled mucous neck cells. The more developed tubules displayed goblet and striated border cells. When the tubules developed further and grew toward the muscularis mucosae, Paneth and argentaffin cells also could be recognized. A similar description of the intestinal tubules has been published (*35*). With progress of the differentiation process, the mitotic activity of the cells of the tubules seemed to decrease.

The fairly similar mucosal changes observed in gastric-ulcer and cancer stomachs contrasted with those of duodenal-ulcer stomachs, together with increased mitotic frequency, may be related to the higher incidence of carcinoma in gastric-ulcer patients (*36*), and it may follow that the gastric cancer originates as a growth in a multicentrically changed mucosa. The last-mentioned hypothesis has been stated before (*37*). The changes thus originating consist of most of the neoplastic-cell types and goblet and striated border cells (*20*).

The higher renewal rate in the gastric mucosa may be a compensatory phenomenon that is caused by increased cell turnover and associated as a reparative process with the restoration of local homeostasis of the mucosa. The changed metabolism of a morbidly changed stomach possibly causes an accelerated consumption and destruction of cells and thus an increased renewal. This can perhaps take place through growth-stimulating factors liberated from cells that are undergoing destruction or have been destroyed. Necrotic and autolytic hepatic tissue distinctly accelerate the liver regeneration of hepatectomized rats (*38*, *39*).

In regard to the relationship between ordinary and intestinal epithelium in the stomach, stimulation of the mitotic activity of the rat stomach has been observed in both stomach and intestine after injection of homogenates (*40*). A greater than normal number of mitoses has been established in the liver of rats growing a transplanted tumor (*41*). An opposite effect has been observed in the cornea and duodenum (*42*), which, however, could not be confirmed (*43*). In regard to the specificity of growth factors, reference is made to the review of Paschkis (*44*).

If growth factors of this type appear in the mucosa it is possible that they also influence the differentiation of cells and lead to the occurrence of the various, more poorly differentiated cell forms found in abundance in the gastric-ulcer and cancer cases.

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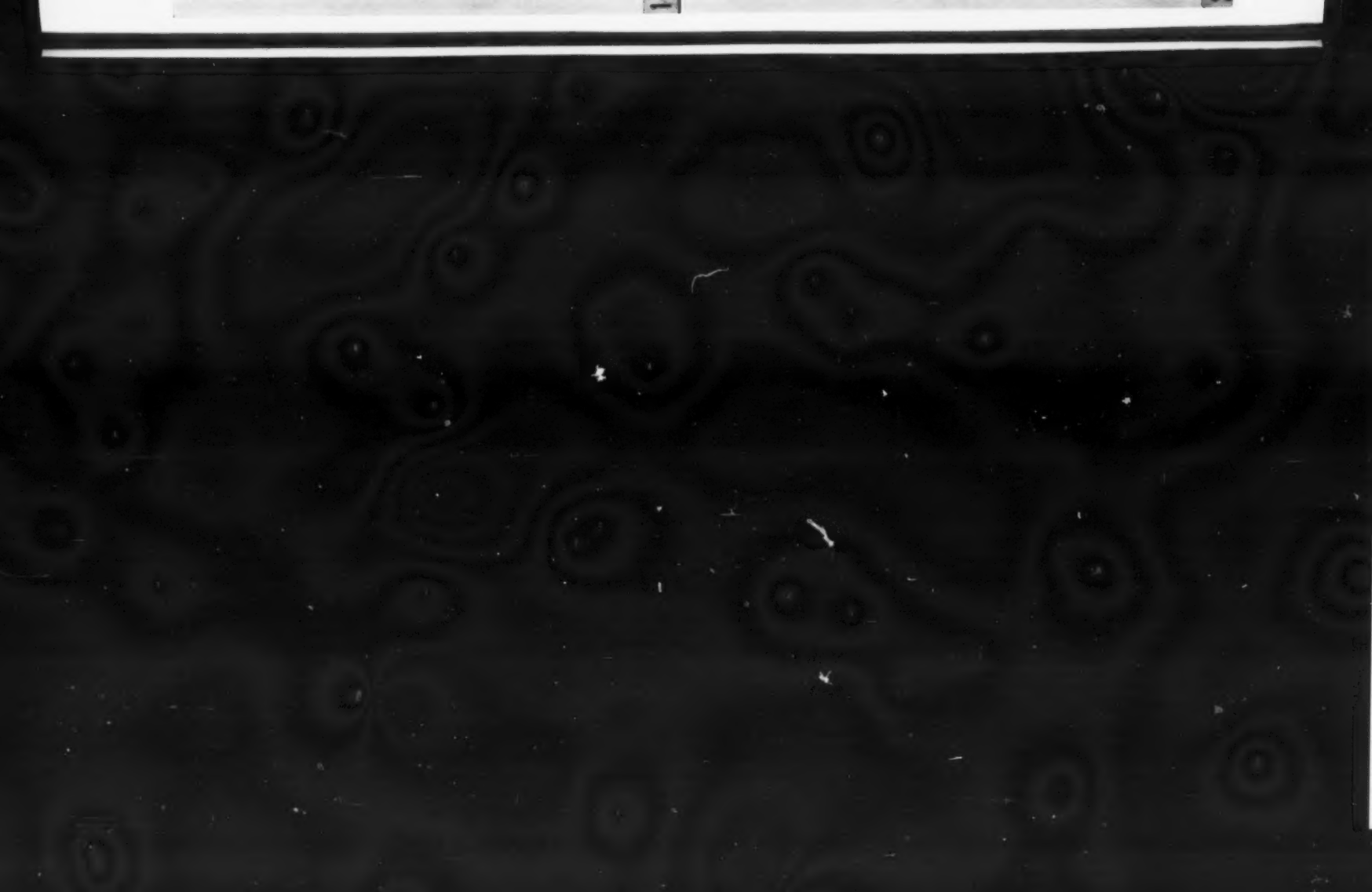
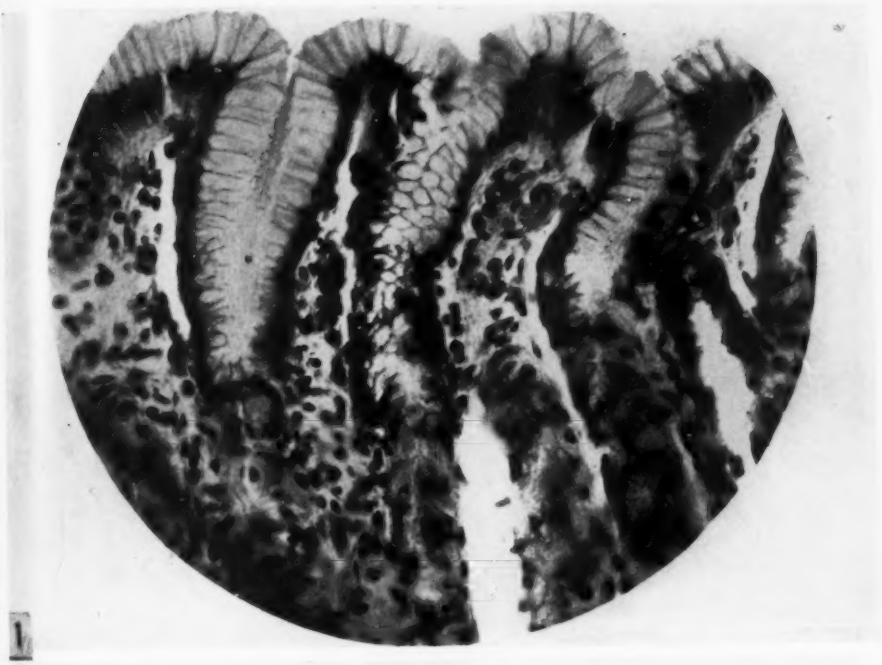
Plate 136

Figure 1.—Normal-looking superficial epithelium in body mucosa of duodenal-ulcer stomach. × 400

Figure 2.—Disturbed superficial epithelium in body mucosa of gastric ulcer stomach. × 400





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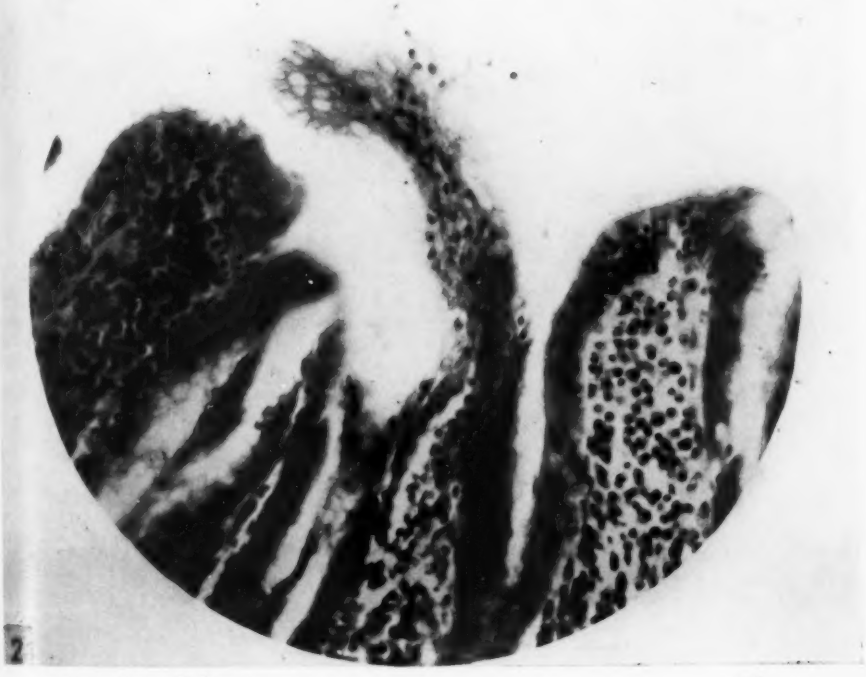

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PLATE 137

FIGURE 3.—Disturbed superficial epithelium in body mucosa of gastric-cancer stomach. × 400

FIGURE 4.—Lengthened mitotically active part in body gland of gastric-ulcer stomach. × 400

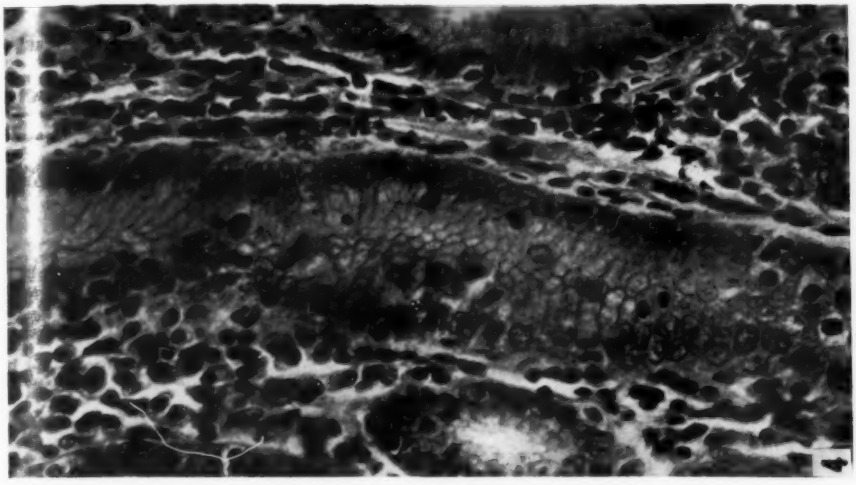
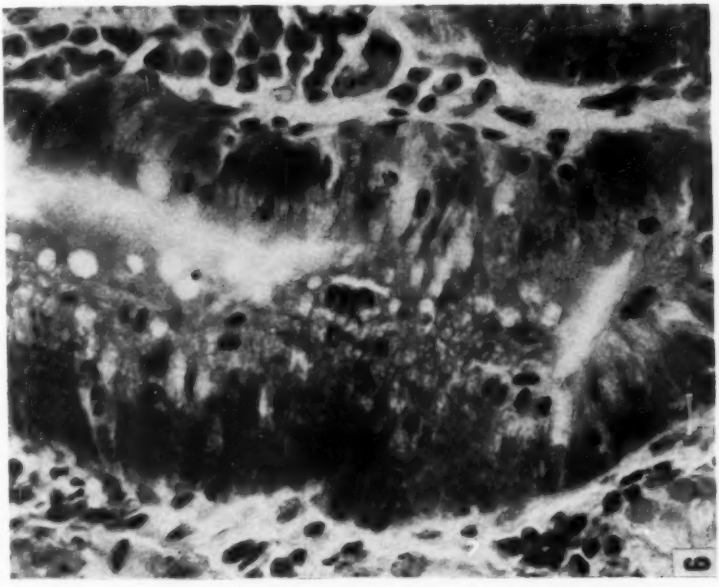


PLATE 138

FIGURE 5.—Increased mitotic activity in an unusual tubule in mucosa in a case of gastric ulcer.

FIGURE 6.—Intestinal crypt in the development with high mitotic activity in gastric mucosa of gastric-ulcer stomach.



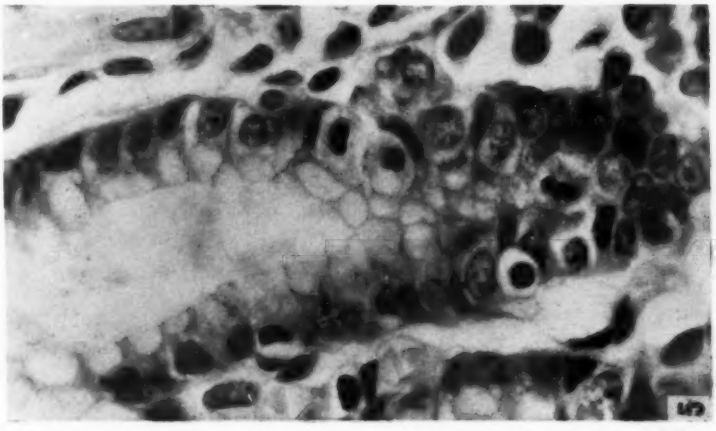


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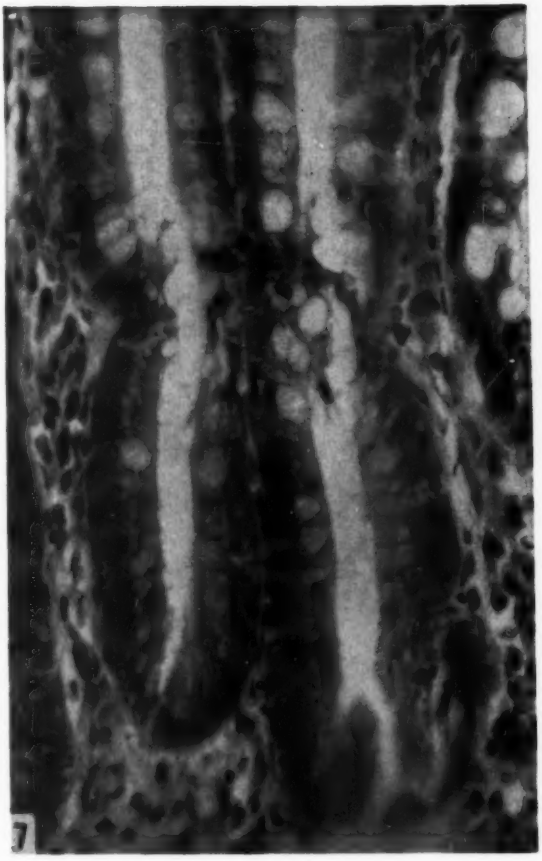
PLATE 139

FIGURE 7.—Fully developed intestinal crypts with scanty mitoses in gastric mucosa of cancer stomach. × 400



Gm Factors in Normal γ-Globulin Fractions, Myeloma Proteins, and Macroglobulins [1]

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SUMMARY

The Gm groups in man are genetically determined properties of normal serum γ-globulins. In the present study, Gm factors (a), (b), and (x) were measured in fractions of normal serum γ-globulin obtained by anion-exchange (DEAE) cellulose chromatography. Gm factors appeared to be present in the same proportion in all the normal human γ-globulin fractions, with ultracentrifugal sedimentation coefficient of about 6.6S. However, the normal fraction, composed primarily of 18S globulin, had no detectable activity. Myeloma proteins, classified immunoelectrophoretically as γ-type and having a single 6.6S component in the ultracentrifuge, varied in their Gm activities. Two Gm factors were undoubtedly present on one such protein. Other myeloma proteins appeared to have only one of the two or three Gm factors present in normal γ-globulins. Several myeloma proteins showed activity equal to that of normal γ-globulin for one Gm factor, but other Gm factors were found at very low levels. The quantitative differences in Gm activity of the individual γ-type myeloma globulins are believed to reflect qualitative differences in the proteins. Myeloma proteins classified immunoelectrophoretically as β_{2A}-type and having multiple (6.6S, 9S, 11S, and 13S) components in the ultracentrifuge did not have demonstrable Gm factors. The γ-18S macroglobulins (β_{2M} on immunoelectrophoresis) similarly were without Gm factors. Reduction of these macromolecules to smaller units with sedimentation coefficients of about 6.6S did not uncover masked or latent Gm activity. The finding of Gm activity on γ-myeloma globulins indicates that malignant plasma cells retain certain genetic features of normal plasma cells.—J. Nat. Cancer Inst. 27: 973–981, 1961.

HERITABLE DIFFERENCES in human γ-globulin were discovered by Grubb (*1*) and have been termed Gm groups. Three factors which certainly belong to the same genetic system are now known: Gm(a) (*1*), Gm(b) (*2*), and Gm(x) (*3*). That the Gm(a) factor behaves as if it were

[1] Received for publication July 5, 1961.

[2] We wish to thank Dr. J. H. Humphrey for suggesting the possibility that we should collaborate in this work and Dr. R. A. Kekwick for his constructive criticism of our manuscript. We are indebted to Dr. W. R. M. Alexander, Dr. I. Dunsford, Dr. M. Harboe, and Dr. A. Steinberg for most generous supplies of reagents and to Miss Mary E. Lawrence for assistance in preparing the protein fractions.

[3] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

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controlled by a gene which is expressed in the heterozygote has been supported by numerous family studies in Europeans (*3–8*). The Gm(b) factor is probably controlled by an allele at the same Gm locus (*3*, *9*). The Gm(x) factor almost always occurs in association with Gm(a) and may be controlled by a third allele Gm^{ax}. In Negroes the situation appears to be somewhat different, since Steinberg, Stauffer, and Boyer (*10*) have good evidence of an allele Gm^{ab} which controls the presence of *both* Gm(a) and Gm(b) factors in the serum; this hypothesis has been supported by family studies in Africans (*9*). Thus most of the work on the Gm groups has been directed toward an elaboration of the number of factors involved and at the development of the pattern of inheritance of these factors. Grubb (*11*) identified the Gm factors as properties of human γ-globulins. In the rabbit, γ-globulin allotypes have been described (*12–14*) which appear to be analogous in many features to the Gm factors in man.

The γ-globulins are now recognized as being composed of three groups of proteins, usually designated the 6.6S or 7S γ-globulins, the β_{2A}-globulins, and the γ-macroglobulins. Each of these groups consists of a family of related but not identical protein molecules. Little is known of the genetic control of the individual molecules within these large groups, though this has been the basis of much speculation in the past few years, particularly from the standpoint of the antibody properties of γ-globulins (*15*).

Where two Gm factors are present among the γ-globulins it is not known, for example, whether all γ-globulin molecules carry both Gm factors or whether some molecules have one factor, some the other or, perhaps, none. The present study was undertaken to determine which group (γ, β_{2A}, or macroglobulin) of serum proteins formed in plasma cells possesses Gm activity, and to determine if electrophoretically different subgroups of normal 6.6S γ-globulin had the same level of activity. Individual myeloma proteins and the macroglobulins of Waldenström's macroglobulinemia are relatively discrete groups of γ-, β_{2A}-, or macroglobulin molecules and represent an opportunity to compare Gm activities on proteins with a minimum of heterogeneity. We hoped to establish whether two or more Gm factors were present on such molecules. Also, the Gm properties of myeloma proteins were compared with normal γ-globulins in the same patient to determine the similarity and possible differences between proteins formed in normal plasma cells and those formed in malignant plasma cells.

MATERIALS AND METHODS

Serums from normal subjects were stored at −20° C until used. Serums from patients with multiple myeloma or macroglobulinemia had been stored from 6 months to 3 years at −10° C and the frozen serums were sent by air from Bethesda to London. Purified fractions were similarly transported. The properties of the anomalous proteins in these serums are presented in detail elsewhere (*16*).

Protein fractionation.—Serum was fractionated into albumin, α_1-, α_2-, β-, and γ-globulin fractions by zone electrophoresis on polyvinyl chloride particle blocks. Fractions were concentrated to approximately the original serum volume and dialyzed against saline prior to analysis for Gm groups. The γ-globulins prepared by block or curtain electrophoresis were further fractionated on columns of DEAE-cellulose (*17*). Samples were equilibrated against 0.02 M phosphate *p*H 8 buffer and applied to columns containing 1 g adsorbent per 10 to 20 mg of protein. Elution was achieved by a gradient of increasing ionic strength to 0.30 M phosphate *p*H 8. The effluent fractions were concentrated and dialyzed against *p*H 7.4 phosphate-buffered saline prior to testing. The protein content was determined by the biuret technique (*17*) or by measurement of the optical density at 280 mμ, with the use of an extinction coefficient ($E^{1\%}_{1\ cm}$ 280) of 12.5 to calculate protein concentrations in mg per ml. Purified myeloma proteins and macroglobulins were made by preparative electrophoresis and chromatography and characterized by electrophoretic, ultracentrifugal, and immunochemical analyses (*16*, *18*). Immunoelectrophoresis, with antiserums specific for γ-, β_{2A}-, or γ-macro (β_{2M})-globulins, permitted antigenic classification of the anomalous proteins (*18*).

Technique for Gm factor estimation.—The technique used was essentially the disagglutination method (*8*). A series of doubling dilutions of the serum or fraction under test was added to sensitized red cells agglutinated by suitable rheumatoid arthritic serums. Great care was taken to make the tests as standard as possible; O, R_1r red cells were used throughout and the reagents used are shown in table 1. Gm titers of ½ were not regarded as positive identification of reactivity since nonspecific inhibition may occur, especially under conditions used for Gm(b) identification where a titer of ½ may not be specific. The reason that the dilution of 265 R.A. and Bomb serum sometimes had to be decreased could be due to a loss of avidity because of repeated thawing and freezing of one particular stock tube. In the case of Pat, one sample was used at ⅒ and a second sample from the same donor was used at ⅛.

TABLE 1.—Reagents used for Gm estimations (vols in ml)

Factor	Cell sensitization anti-D		Saline	Packed cells	Rheumatoid serum
Gm(a)	She 1386	0. 1	3. 0	0. 1	265 R.A. 1/100–1/200
Gm(b)	VIK	0. 1	1. 0	0. 1	Bomb 1/10–1/20
	She 2269*	0. 1	1. 0	0. 1	
Gm(x)	Ask	0. 1	1. 0	0. 1	Pat 1/5–1/10

* When the supply of VIK was exhausted, 2269 had to be used.

RESULTS

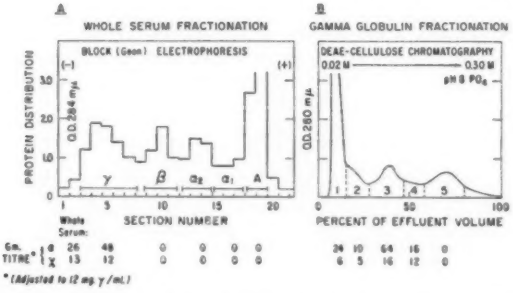
Normal Serum Fractions

Gm activity was in the γ-globulin fractions of two serums subjected to zone electrophoresis (table 2 and text-fig. 1A). All the Gm activities in

TABLE 2.—Gm activity in normal γ-globulins

	γ (mg/ml)	Gm titer* a	b	x
Serum H	12	32	32	32
Serum F	15	32	0	16
Electrophoretic fractions of serum				
Serum H:				
Albumin		0	0	0
α_1		0	0	0
α_2		0	0	0
β		1	1	1
γ	2.5	4	1	32
Serum F:				
Albumin		0	0	0
α_1		0	0	0
α_2		0	0	0
β		0	0	0
γ	8	32	0	8
Chromatographic fractions of normal γ-globulins				
H γ (1)	9	16	8	32
(2)	5	8	4	16
(3)	12	8	16	16
(4)	5	4	2	8
(5)	3	0	0	0
F γ (1)	8	16	0	4
(2)	5	4	0	2
(3)	6	32	0	8
(4)	6	8	0	1
(5)	5	0	0	0

* Expressed as reciprocal of titer.



TEXT-FIGURE 1.—Distribution of Gm factors in serum protein fractions obtained by block electrophoresis (A) and fractions of γ-globulin obtained by DEAE-cellulose chromatography (B). Electrophoresis of serum was carried out in 0.1 ionic strength Veronal buffer, *p*H 8.6, on polyvinyl particle blocks. γ-Globulins prepared by electrophoresis were further fractionated on DEAE-cellulose columns with a gradient elution from 0.02 M to 0.30 M phosphate *p*H 8 buffers (*17*). The Gm titers in each fraction were adjusted to a γ-globulin concentration of 12 mg per ml.

serum were accounted for by the γ-globulins except for trace amounts of activity among the β-globulins of one serum which may have been due to small amounts of γ-related proteins of β-electrophoretic mobility or to contamination with small amounts of true γ-globulin.

γ-Globulin Fractions

γ-Globulins obtained by preparative zone electrophoresis were further fractionated on columns of anion exchange (DEAE) cellulose (text-fig. 1B). Gm activity was found in fractions 1 to 4, which are composed of γ-globulins with sedimentation coefficients of 6.6S. In contrast, fraction 5 containing primarily 18S γ-macroglobulin had little or no Gm activity. All the Gm groups present in the whole serum were in the chromatogram fractions of the 6.6S γ-globulins and in about the same proportion per mg of protein.

γ-Myeloma Proteins

Myeloma proteins were isolated from 7 serums by preparative electrophoresis and DEAE-cellulose chromatography. These myeloma proteins have a single component in the ultracentrifuge with a sedimentation coefficient of about 6.6S, are of γ-electrophoretic mobility, react as γ-type proteins on immunoelectrophoresis, and have a relatively low hexose content. All but one of these proteins showed some Gm activity and several myeloma proteins had two Gm factors (table 3).

Titers ranging from ½ to $\frac{1}{128}$ were estimated for the Gm(a) factor. The range for Gm(x) and Gm(b) was less marked because myeloma proteins with high titers for these factors were not found. Most of the titers observed for myeloma proteins were lower per mg of protein than those for purified normal γ-globulin fractions (*cf.* tables 2 and 3).

Individual myeloma proteins often differed in the activity of various Gm groups. R.P. myeloma protein, for instance, had a Gm(a) titer of $\frac{1}{128}$, but Gm(b) and Gm(x) titers of only ½ and ¼, respectively. In contrast A.M. myeloma protein had Gm(a) and Gm(x) titers of $\frac{1}{32}$ and the Gm(b) titer was only ¼.

β_{2A}-Myeloma Proteins

These myeloma proteins characteristically have several components (6.6S–13S) in the ultracentrifuge, fast γ- or β-globulin zone electrophoretic mobility, the immunoelectrophoretic properties of β_{2A}-globulins, and a higher hexose content than the γ-myeloma proteins. Three myeloma proteins of this type were studied and none had detectable Gm activity of any type. The Gm (a) and (b) activity detected in the serum of the 2 Negro subjects, J.L. and V.H. (table 3), is attributable to the normal γ-globulins remaining in the serum.

Macroglobulins

Three purified γ-macroglobulins from patients with primary macroglobulinemia were tested. The macroglobulins had discrete peaks of distribution in the ultracentrifuge, with sedimentation coefficients of 18S, 22S, and 27S. They had hexose contents of about 6 percent and reacted on immunoelectrophoresis as β_{2M}-components. No Gm activity was found in these proteins.

TABLE 3.—Gm titers for myeloma proteins, macroglobulins, and normal serum globulins of myeloma and macroglobulinemic serums

	Observed values, protein concentration (mg/ml)		Gm titer		
	γ-Globulin	Anomalous protein	a	b	x
Myeloma serums with γ-type myeloma proteins					
R.P., myeloma protein		29	128	2	4
Serum		63	128	8	32
A.M., myeloma protein		27	32	4	32
Serum		63	64	16	64
E.J., myeloma protein		28	8	8	4
Serum		36	128	8	16
B.B., myeloma protein		15	8	2	2
Serum		42	64	8	8
R.G., myeloma protein		25	4	2	4
Serum		54	4	4	—
M.B., myeloma protein		11	4	1	0
Serum		55	16	4	8
C.R., myeloma protein		24	1	1	1
Serum		62	8	2	4
Myeloma serums with β_{2A}-type myeloma protein					
J.L., myeloma protein		19	0	0	0
Serum	6	45	16	16	0
H.B., myeloma protein		16	0	0	0
Serum	2	50	0	8	0
V.H., myeloma protein		6	0	0	0
Serum	3	15	2	8	2
Primary macroglobulinemia					
J.W., macroglobulin	—	55	0	0	0
Serum	3	74	0	0	0
Normal γ	3	—	0	2	0
I.P., macroglobulin	—	34	0	0	0
Serum	2	68	4	8	4
E.C., macroglobulin	—	15	0	0	0
Macroglobulin	—	55	0	0	0
Serum	2	60	4	4	4
Normal γ	4	—	1	16	1

Dissociation of β_{2A}-Myeloma Proteins and Macroglobulins

The 18S γ-macroglobulins are believed to be polymers of 6.6S molecules (*19*), and the β_{2A}-myeloma proteins consist, at least in part, of aggregates of 6.6S molecules (*16*). Because the macroglobulins and the β_{2A}-proteins were without Gm activity, whereas the 6.6S γ-myeloma proteins and normal γ-globulins did show Gm activity, depolymerization to 6.6S units of three macroglobulins and one β_{2A}-myeloma protein was carried out. Preparations of purified protein were divided into three aliquots. One portion was treated with the sulfhydryl compound, mercaptoethanol, and then allowed to reaggregate by the removal of the mercaptoethanol through dialysis against *p*H 7.4 phosphate-buffered saline. A second aliquot was reduced to 6.6S molecules by mercaptoethanol dialysis and then fixed as 6.6S molecules by reaction with iodoacetate according to the technique of Deutsch and Morton (*19*). A third aliquot was dialyzed against buffered saline as a control. Ultracentrifugal analysis confirmed

that the expected changes had occurred. After thorough dialysis against *p*H 7.4 phosphate-buffered saline, all samples were retested for Gm activity, but there was no inhibition of the Gm(a), (b), or (x) factors, as shown in table 4.

TABLE 4.—Gm inhibition titers before and after treatment with mercaptoethanol *

			Gm		
			a	b	x
Macroglobulins E.C., J.W., and I.P. and β_{2A}-myeloma protein J.L.		(C)†	0	0	0
		(M)†	0	0	0
		(I)†	0	0	0
Control experiments with γ-myeloma globulins	A.M.	(C)	32	4	16
		(M)	16	8	16
		(I)	8	4	8
	R.K.	(C)	64	2	1
		(M)	32	2	1
		(I)	32	2	1

*All samples tested at 10 mg per ml protein concentration.

†C = Dialyzed against saline; M = treated with mercaptoethanol and then dialyzed against saline; I = as M and then treated with iodoacetate before saline dialysis (*19*).

Comparison of M Proteins and Normal γ-Globulins in the Same Serums

The Gm activity of normal (nonmyeloma) γ-globulins in the myeloma and macroglobulinemic serums was measured in several instances by isolation of a normal 6.6S γ-globulin chromatogram fraction separate from the anomalous globulin (*18*). In most serums, however, the Gm properties of the normal γ-globulins were assessed by a comparison of the Gm activities of whole serum with those of the isolated myeloma protein. Activity of whole serum that could not be accounted for by myeloma protein activity was presumed to be a property of normal γ-globulins. Gm activity was confined to the normal γ-globulin in serums with macroglobulinemia or with β_{2A}-myeloma proteins. The low titers of Gm activity are compatible with the low levels of normal γ-globulins in these serums.

DISCUSSION

The molecular configurations responsible for the Gm factors (a), (b), and (x) are restricted to the γ-globulins, which typically are 6.6S γ-globulins and account for the majority of proteins in the γ-electrophoretic region. Electrophoretic subfractions of this group of γ-globulins appeared to have the same Gm properties. The question, however, of whether single molecules had more than one Gm factor could not be answered by study of normal γ-globulins, for even the chromatographic fractions are markedly heterogeneous. Studies of γ-type myeloma proteins revealed that these globulins may contain two or three of the Gm factors. Although myeloma proteins are not always homogeneous (*16*) they represent

the most discrete γ-globulin available for study, and the finding of several Gm factors with individual myeloma globulins supports the view that Gm(a), (b), and (x) may be properties of the same γ-globulin molecule.

These observations do not indicate whether all γ-globulin molecules contain each of the Gm factors found in a serum. While the γ-myeloma globulins accounted for much of the Gm activity in their serums, they frequently did not account for all the activity, and most serums contained some Gm factors not represented at all or not strongly represented by the myeloma protein molecules. This could be interpreted as a loss of specificity (or loss of antigenicity if the Gm factors can be established as antigens) in the product of the malignant cells. The phenomenon of antigenic loss of neoplastic cells as compared with the corresponding normal tissue has been demonstrated by Kay and Wallace (*20*) in tumors arising from urinary epithelium in man.

Macroglobulins and β_{2A}-myeloma globulins did not have reactive Gm sites, though Gm factors were present in the serum of these subjects in the remaining normal 6.6S γ-globulins. Since there is good reason to believe that the β_{2A}-globulins, macroglobulins, and 6.6S γ-globulins are made in plasma cells or lymphocytoid cells, it is apparent that these cells differ in their ability to confer Gm properties on the molecules they synthesize. How closely the genetic factors determining Gm properties are related to the factors determining other structural features of the proteins produced in plasma cells will have to be determined by additional observations.

The observations of Gm activity in γ-myeloma proteins shed some light on the genetic status of the malignant plasma cells and indicate that these cells have retained the capacity of normal plasma cells to transmit genetic information to γ-globulin. Since malignant plasma-cell tumors probably are derived from a single clone of normal plasma cells, the evidence that γ-myeloma proteins may have two or more Gm factors would indicate that at least some normal plasma cells may also have a full capacity to confer Gm factors on their normal γ-globulin products. The observations on transmission of Gm genetic information relate as well to the problems of transmission of information regarding antibody synthesis. If malignant plasma cells can transmit genetic information in the same manner as normal plasma cells and if antibody characteristics are genetically determined (*15*), myeloma proteins with antibody activity are to be expected. Although no myeloma protein has yet been shown to have specific antibody activity, continued search for such a property seems warranted.

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Chromosome Studies in Human Leukemia. I. Acute Leukemia in Children [1, 2, 3]

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SUMMARY

Metaphase chromosome number and morphology were investigated in short-term cultures of leukocytes from the peripheral blood of 7 children with acute leukemia, ranging in age from 3 months to 11 years, and of 2 nonleukemic children, both 6 years old. Leukocytes from the 2 nonleukemic children showed no significant departure from the diploid chromosome number ($2n = 46$). In 10 karyotype analyses on each of the 2, no detectable structural changes were present in the chromosomes. In all the leukemia cases, the majority of cells also had 46 chromosomes. From 5 to 15 karyotype analyses were made in each case, and the *majority* of these cells had *numerically and morphologically normal* chromosome complements. Such structural changes as were present revealed no characteristic pattern. In the 3 cases which had undergone extensive therapy, and in which symptomatic disease had been present for from 6 months to 3 years, the incidence of karyotype change was higher than in the remaining 4 cases, 3 of which were untreated and the fourth of which had been treated for only 5 days. These data are interpreted to indicate that the observed chromosome changes are not causally related to the onset of the disease.—J. Nat. Cancer Inst. 27: 983-1011, 1961.

THE CHROMOSOMES in human leukemia have for some decades been investigated with older techniques. Such work [*see* Nowell and Hungerford (*1*) for references] is now largely of historical interest. In 1958, reinvestigation of this problem was begun with modern techniques (*2*, *3*).

[1] Received for publication May 5, 1961.

[2] This study constituted a portion of a dissertation in zoology presented to the Faculty of the Graduate School of Arts and Sciences of the University of Pennsylvania in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[3] Supported in part by a terminal year predoctoral research fellowship, CF-11,168, from the National Cancer Institute, National Institutes of Health, Public Health Service, and earlier by a junior research fellowship from The Institute for Cancer Research, Philadelphia. Additional support was provided by research grant C-1613 from the National Cancer Institute, National Institutes of Health, Public Health Service.

[4] The author wishes to express his appreciation to Dr. Jack Schultz, in whose laboratory this investigation was carried out, for valuable advice during the course of the work and for helpful criticism during the preparation of the manuscript; to Dr. Peter C. Nowell, who several years ago first called the author's attention to the potential advantages of employing cultures of leukocytes from peripheral blood for human chromosome studies, for collecting and culturing the material used in this study, and for preparing the case reports; to Dr. Arthur E. McElfresh, Dr. Lester Weiss, and Dr. Irving J. Wolman, who made clinical material available; and to Miss Sherrod Daugherty, Miss Sandra S. Ferry, Mrs. Elizabeth Krohnert, and Miss Joyce Wagner for able technical assistance.

A number of reports has followed, and these will be discussed later in the present paper.

A major question concerning the chromosomes in leukemic and other neoplastic diseases is whether detectable chromosome changes are present initially or whether the chromosome changes observed in many mammalian neoplasms are secondary phenomena. If such changes are present and presumably etiologically related to neoplasia, it would be of great interest to determine their character and specificity, if any.

To date, only two published studies have included any investigation of the chromosomes in childhood leukemia (*see* Discussion), and there are still relatively few studies on the chromosomes of leukemia in adults. The analysis of acute leukemia in children to be presented here complements those already made (*1*, *4*) and in progress, and will be discussed in contrast to the findings in chronic granulocytic leukemia and in context with the findings of other workers in the field.

MATERIALS AND METHODS

Chromosome counts and karyotype analyses were carried out on metaphases occurring in short-term cultures of leukocytes from the peripheral blood and in one case on metaphases from a bone marrow biopsy as well. Methods currently in use for the separation, culture, and cytological preparation of these cells have already been published in detail (*5*).

In the construction of the karyotype analyses, the chromosomes were classified according to the Denver system of nomenclature.[5]

Criteria in addition to those given in the Denver report have been noted in these preparations (Hungerford: Doctoral dissertation, University of Pennsylvania, February, 1961), and at present it is believed possible to recognize individually all chromosomes in the best metaphases except #4 and 5 and 11 and 12. These are recognized as 2 discrete groups.

CASE REPORTS

122T.—G.L., a 3-month-old white male, was admitted to Children's Hospital, Philadelphia, March 30, 1960, with a history of normal development until 1 week before admission, when marked pallor and an abdominal mass were noted. The white blood cell count was abnormal and hepatosplenomegaly was present. Bone marrow diagnosis: "acute lymphocytic leukemia." Treatment with steroids and 6-mercaptopurine was unsuccessful and he died April 26, 1960. Blood and bone marrow specimens for chromosome study were obtained March 31, 1960, immediately before therapy was started; at that time the white blood count was 16,800 with bands and segmenters 30 percent, lymphoblasts 1 percent, and lymphocytes 69 percent (19% abnormal).

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[5] "A proposed standard system of nomenclature of human mitotic chromosomes" has appeared in the following places, among others: Lancet i: 1063-1065, 1960; Am. J. Human Genet. 12: 384-388, 1960; J.A.M.A. 174: 159-162, 1960.

124T.—J.M., an 11-year-old white male, whose case was diagnosed as acute lymphocytic leukemia at St. Christopher's Hospital, Philadelphia, in May, 1957, was first treated with methotrexate (4-amino-N^{10}-methylpteroylglutamic acid, Lederle) and attained a remission which lasted until July, 1958. A relapse at that time responded to 6-mercaptopurine and a second remission occurred. In January, 1960, he relapsed again and was treated with methotrexate and steroids, but his disease responded poorly to therapy. His condition gradually deteriorated and he died May 4, 1960. A blood sample for chromosome study was obtained April 5, 1960; at that time the white count was 7,000 with bands and segmenters 22 percent, lymphoblasts 13 percent, and lymphocytes 65 percent.

135T.—M.G., a 6-year-old white female, whose case was diagnosed as acute stem-cell leukemia at St. Christopher's Hospital, Philadelphia, in July, 1957, was treated briefly with steroids and then with methotrexate, and a remission was attained. On maintenance therapy with methotrexate, the remission lasted until July, 1960, when, because of signs of relapse, her medication was changed to 6-mercaptopurine and steroids. She failed to respond and died September 1, 1960. A blood sample for chromosome study was obtained July 19, 1960; at that time the white blood count was 8,000 with bands and segmenters 2 percent, lymphocytes 29 percent, and blasts 69 percent.

This patient was one of 6 siblings. A brother, 6 years older, died at age 4½ years of congenital heart disease (septal defect). A brother, age 2 years, is a mongoloid idiot. Three siblings (2 males, ages 4 and 11 years, and 1 female, age 8 years) are apparently normal. The father, age 38, and the mother, age 34, are unrelated; no historical data which appeared significant were obtained from either parent.

136T.—R.R., a 3-year-old white male, was admitted to St. Christopher's Hospital, Philadelphia, July 19, 1960, because of abnormal hematological findings (anemia, thrombocytopenia, and circulating blasts) noted at another hospital. A bone marrow examination revealed acute stem-cell leukemia. He was treated with 6-mercaptopurine and steroids for 4 weeks and attained a remission which continues to date (Nov. 1, 1960). A blood sample for chromosome study was obtained July 20, 1960, before therapy was started; at that time the white blood cell count was 5,000 with segmenters 1 percent, lymphocytes 59 percent, and blasts 40 percent.

137T.—R.R., a 5-year-old white male, whose case was diagnosed as acute granulocytic leukemia at St. Christopher's Hospital, Philadelphia, in February, 1960, was treated with methotrexate and steroids and attained a remission which lasted until July, 1960. Relapse at that time was treated with 6-mercaptopurine until September and then with steroids. His disease responded poorly to treatment and he remains in relapse at the present time (Nov. 1, 1960). A blood sample for chromosome study was obtained July 20, 1960; at that time the white blood cell count was 9,400 with blasts and promyelocytes 16 percent, myelocytes 2 percent, metamyelocytes 13 percent, bands and segmenters 31 percent, and lymphocytes 38 percent.

138T.—L.L., a 5-year-old Negro male, was admitted to St. Christopher's Hospital, Philadelphia, July 23, 1960, because of fever and weakness of 1 week's duration. Bone marrow examination revealed acute lymphocytic leukemia. He was treated with methotrexate and attained a remission which has lasted to date (Nov. 1, 1960). A blood sample for chromosome study was obtained July 25, 1960, before therapy was started; at that time the white blood cell count was 16,800 with bands and segmenters 14 percent, lymphoblasts 56 percent, and lymphocytes 30 percent.

146T.—T.M., an 8-year-old white male, was admitted to Abington Memorial Hospital, Abington, Pennsylvania, September 19, 1960, with a history of pallor and weakness for 1 week. The white blood count was 4,400 and the bone marrow diagnosis "acute lymphocytic leukemia." Treatment with 6-mercaptopurine was started September 21, 1960. A blood sample for chromosome study was obtained September 26, 1960; at that time the white blood cell count was 3,200 with bands and segmenters 25 percent, lymphoblasts 34 percent, lymphocytes 37 percent, and monocytes 4 percent.

RESULTS

The total number of chromosome counts and the chromosome number for each count are given in table 1. In addition there is the total number of karyotype analyses performed on each case along with the number in which morphological changes were observed.

The peripheral blood of 2 nonleukemic children, whose ages are within the range represented by the leukemic children in this study, was also examined. The results are presented in table 2.

TABLE 1.—Chromosome counts and analyses in 7 cases of acute leukemia in children

Case	Counts								Total counts	Total analyses	Abnormal karyotype*
	40	42	43	44	45	46	47	>47			
122T (blood)						34			74	8	1
(marrow)						40				7	1
124T (blood)			2	1	7	69	1		80	11	3
135T (blood)			2	1	3	45		1†	52	5	1
136T (blood)	1	1	2	3	8	62			77	10	0
137T (blood)			1		6	60	1		68	7	3
138T (blood)					3	65			68	8	1
146T (blood)					6	66			72	7	1

* *See* text and figures for further details.
† Fifty-one chromosomes.

TABLE 2.—Chromosome counts and analyses from leukocytes in the peripheral blood of 2 nonleukemic children

Case	Age, sex	Counts					Total counts	Total analyses	Abnormal karyotype
		43	44	45	46	4*n*			
148H	6 years, female	1		5	44		50	10	0
149H	6 years, male			1	48*	1	50	10	0

* One of these metaphases included a small fragment, but could not be analyzed.

As described in the case histories, 4 of the 7 leukemic children had been treated. Table 3 demonstrates the relationship among observed frequency of abnormal karyotypes, duration of illness, and history of therapy.

It will be seen from the foregoing that the majority of karyotypes as well as chromosome numbers from the leukemic children are normal. A description of the chromosome analyses in each case follows.

In these accounts, all cells analyzed had 46 chromosomes unless specifically stated otherwise. These metaphases were selected for analysis solely on the basis of their clarity. They represent largely, if not entirely, the first mitoses *in vitro* (*see* Discussion).

TABLE 3.—Relation of time course of disease and amount of therapy to observed frequency of abnormal karotypes in leukemic children

Case	Duration of symptomatic disease and amount of therapy	Frequency of abnormal karyotypes
124T	Three years, treated	3/11
135T	Three years, treated	1/5
137T	Six months, treated	3/7
146T	Eleven days, treated for 5 days	1/7
122T	One week, no treatment	2/15
136T	Short, no treatment	0/10
138T	One week, no treatment	1/8

In case 122T, 8 analyses were done on bone marrow (this is the only case in which bone marrow was obtained), of which 7 were normal; 7 were done on peripheral blood, of which 6 were normal. The chromosome aberrations in the two abnormal cells were identical (fig. 1): One of the two #6 chromosomes is missing, and an abnormal chromosome similar to a normal #18 is present; also, one of the two #16 chromosomes is abnormally long. These changes could be interpreted as a 6 to 16 translocation (perhaps reciprocal) plus a deletion. The X chromosome may be abnormal.

In case 124T, 11 analyses were done, of which 8 were normal. The chromosome aberrations in 2 of the 3 abnormal cells were identical and involved one of the #14 chromosomes being replaced by an apparently abnormal longer chromosome with an atypical arm ratio (fig. 2). In the third abnormal cell, changes involved the X and both #6 chromosomes (fig. 3).

In case 135T, 5 analyses were done, of which 4 were normal. In the single abnormal cell (fig. 4), 2 chromosomes in the 7 to 12 group showed changes. Two normal 11 to 12 chromosomes are missing and are replaced by 2 abnormal chromosomes. In one, the short arm is increased in length to such an extent that the centromere is in a more median position than is normal for any chromosome in this group; in the other, the arm ratio is much too high for any chromosome in the group. A small change in the two #10 chromosomes is also suggested. A variety of mechanisms may have been responsible for these changes.

In case 136T, 10 analyses were done, all of which were normal (fig. 5).

In case 137T, 7 analyses were done, of which 4 were normal. The 3 abnormal cells are markedly dissimilar. In one (fig. 6), the normal X chromosome was not present, and in its place there was a larger chromosome (intermediate in length between chromosomes #1 and 3) with a median centromere. No other chromosome in the complement of this cell differed from normal. In the second (fig. 7), 1 chromosome #20 was missing, 1 of the 4 group 21 to 22 chromosomes had abnormally large satellites and stalks, and an abnormal chromosome somewhat similar to chromosome #18 was present. One chromosome #6 may be abnormal. In the third (fig. 8), there were 47 chromosomes; one #6, one #10, and both #17 chromosomes were missing. Three apparently normal chromosomes,

any or all of which could be X, were present. In addition there were 3 abnormal chromosomes similar in morphology to the normal chromosomes in group 13 to 15.

In case 138T, 8 analyses were done, of which 7 were normal. In the single abnormal cell (fig. 9), one chromosome #16 was missing, and there was present a chromosome similar to but larger than the normal 21 to 22 chromosomes. This change may represent a chromosome #16 deletion.

In case 146T, 7 analyses were done, of which 6 were normal. The single abnormal cell (fig. 10) showed a change in 2 of the 6 group 13 to 15 chromosomes. One was nearly twice too long, and the other was represented by a minute of less than half the size of the smallest normal autosome, which strongly suggests that these are the products of a reciprocal translocation.

DISCUSSION

Several significant findings have emerged from the present study. One is that there is no change in chromosome *number* in the vast majority of the cells observed.

A second is that while there is a variable frequency of aberrant karyotypes among the cells analyzed in some cases, there was in general no consistent pattern of change in the leukemic cells of these children. This finding is in contrast with observations in chronic granulocytic leukemia to be described.

Thirdly, the frequency of cells showing chromosome aberrations appears to bear a direct relationship to the duration of illness and the history of therapy. The 3 cases of longest duration and with the most intensive therapy (124T, 135T, and 137T) had approximately twice the frequency of karyotype change as the 4 remaining cases, 3 of which (122T, 136T, and 138T) had received no treatment, while the fourth (146T) had undergone therapy for only 5 days.

The results published to date on chromosomes in human leukemia show a number of discrepancies. In the review which follows, however, it will be seen that in general the human leukemias are distinguished by a more nearly normal chromosome complement than are those which have been described in the cells of other human neoplasms [*see, e.g.*, Makino *et al.* (*6*)]. While the present discussion will deal specifically with human leukemias, Bayreuther's findings (*7*), that the chromosome number in a variety of early primary neoplastic growth is diploid, require a reappraisal of the results from other cytological investigations of tumors which at the time of analysis were later in the course of their progression.

In the initial report on the human leukemias investigated by modern cytological techniques, Nowell *et al.* (*2*) reported no departure from the diploid number and no gross changes in chromosome morphology in the peripheral blood of 3 adult individuals with acute granulocytic leukemia. Two of these cases were given in greater detail subsequently (*1*).

Shortly afterward, Ford *et al.* (*3*) described results obtained by the use of the bone marrow method on 3 cases of leukemia in humans (ages not

given). In one case of lymphocytic and in another of blast-cell leukemia, there were no evident departures from normal chromosome number and morphology. In the third case, of unspecified type, an abnormal number of 45 chromosomes including one minute was observed. Ford (*8*) has briefly mentioned work with Lajtha on 7 more cases of leukemia. Two of these are stated to be clearly abnormal, 1 with a mode at 48 chromosomes, the other with a mode at 46 but including a minute. No further details were given.

Even greater variability has been described recently from two other laboratories.

Baikie *et al.* (*9*) have investigated bone marrow cells in 5 cases of acute leukemia (type unspecified in 4 cases), 5 cases of chronic myeloid leukemia, 1 case of chronic lymphatic leukemia, and 2 cases of myelomatosis, all in adult humans, the youngest of whom was 41 years old. Only 1 case, specified as acute myelomonocytoid leukemia, in which the peripheral blood picture is described as relatively aleukemic, is given in detail. Preliminary reports are given on the other 10. To summarize their results: In 2 cases of acute leukemia abnormalities of number and morphology were found, in 2 others only morphological abnormalities, and in the fifth no change. No abnormalities of either kind were found in any of the 6 cases of chronic leukemia or in the 2 cases of myelomatosis. Interestingly, in the 1 case reported in detail, both numerical and morphological chromosome changes occurred in the sternal marrow over a period of 8 months, during which time 5 samples were taken for study.

Sandberg *et al.* (*10*) have recently reported their results from the investigation of bone marrow in 7 cases of leukemia. Three of these were acute lymphoblastic leukemias in children of 3½, 12, and 4 years. Counts of 46 were observed in 85 percent, 0, and 26 percent of the cells, respectively. Adult leukemias were represented by 2 acute myeloblastics, 1 chronic lymphocytic, and 1 chronic myelocytic; counts of 46 represented 29, 51, 36, and 10 percent of the cells, respectively. In all this leukemic material, no consistent pattern of chromosome change was described among the aneuploid cells and no analyses of cells having the diploid number were given. These authors feel that the use of short-term cultures, as compared to the direct preparations used by them, prejudices the results in these chromosome studies by allowing normal but not leukemic cells to divide. Ford and Mole (*11*) had earlier expressed a similar concern regarding the negative findings of Baikie *et al.* in chronic leukemia. This matter will come under discussion in the following pages.

Recently, Nowell and Hungerford (*4*) have reported a characteristic change in the karyotypes of leukocytes from the peripheral blood of individuals with chronic granulocytic leukemia. This study is still in progress. In 11 of 12 such cases investigated to date, a characteristic abnormally small chromosome is present. It is apparently 1 of the 4 smallest acrocentric (probably #21) autosomes with a large portion of its longer arm missing. This is the only example thus far of a specific

chromosome change in a mammalian neoplasm. A full report will constitute the second paper in this series. Very recently, the Edinburgh group has published two papers amply confirming this finding in chronic granulocytic leukemia (*12*, *13*). In the latter of these papers, the minute chromosome has been designated "Ph^1." In the same publication, results from a study of acute leukemia in 5 cases of mongolism, 4 of which were in children, are also given. Chromosome abnormalities other than the mongol karyotype were not observed.

Cited briefly by Ferguson-Smith and Johnston (*14*) are the unpublished findings of the latter in 4 untreated cases of acute leukemia and 2 cases of chronic leukemia (types not specified). According to these authors, "The modal diploid number was normal in each, although the proportion of hyperploid cells was much higher than is usually found in normal bone marrow."

The bases of the discrepancies between the findings on peripheral blood and some of the findings on bone marrow are not easily understood. However, the reports concerning aneuploidy must be considered in the light of the following reservations: 1) the possible effects of therapeutic agents, when present and especially when administered over a long period, on some of the cells of the marrow, 2) the possibility that aberrant mitoses are present in the marrow as a result of selective pressure manifested secondarily during the course of the disease, especially if the course has been a long one, 3) the possibility that the products of such mitoses may not be viable in the peripheral blood, and 4) the possibility that some of the abnormal mitoses studied were in cells of types other than those involved in the leukemic condition, and which have been described in normal marrow [*e.g.*, Schwarz (*15*)].

Related to the chromosome studies are the data of Hale and Wilson (*16*), who published a preliminary communication concerning the deoxyribonucleic acid (DNA) content of nuclei in 32 samples from leukemia and related diseases. In cells from the peripheral blood and in the majority of bone marrow samples, the DNA distribution was characteristic of that in nuclei from normal tissues. However, in 2 cases of subacute lymphatic leukemia, 1 of acute monocytic leukemia, and 1 of reticulosarcoma, the DNA distribution was abnormal, *i.e.*, the modal absorbancy was either 1) not characteristic of the diploid amount of DNA, or 2) secondary modes were present outside the extreme limits of the diploid range. Unfortunately for the purpose of the present discussion, the minimum error inherent in cytophotometric measurements of Feulgen-stained nuclei is such that relatively small chromosome changes, easily observed visually, would remain undetected. As an approximation it may be assumed that, in metaphase plates, the length of each chromosome is proportional to the amount of DNA which it contains. If this is true, each of the 4 small acrocentric autosomes (group 21–22), measured in plates not showing pronounced condensation of the chromosomes, represents less than 1 percent of the total DNA content of the nucleus, which easily may be less than the error of the cytophotometric method [*see, e.g.*, Walker and Richards (*17*)].

Although the relationship of the chromosome findings reported here to the etiology of childhood leukemia remains obscure, it can be stated that the present results are certainly not in keeping with the hypothesis that visible chromosome changes are of primary etiological importance. Such changes as those which have been described here are best interpreted as secondary to the onset of the disease. Whatever primary changes have occurred in these cells are apparently not at the level observable by the use of the light microscope and present preparative techniques.

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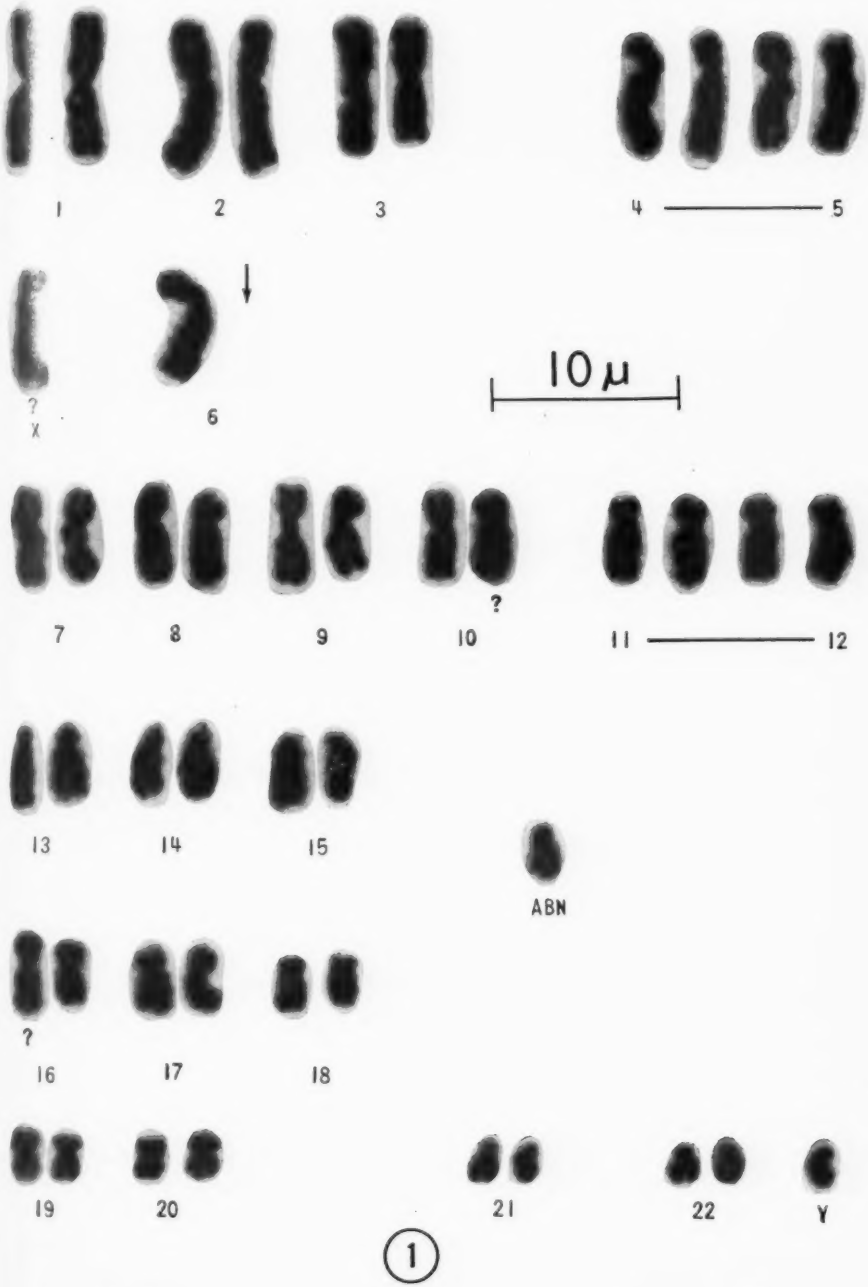
In these karyotype analyses, the chromosomes are classified according to the Denver system (*see* text). All magnifications are the same, and are denoted by the 10 u scale included in figure 1. *Arrows* indicate the positions from which normal chromosomes are missing. "ABN" indicates an abnormal chromosome. A question mark indicates some doubt concerning the classification of the chromosome so signified. Interpretations which are possible for some of the changes in the abnormal karyotypes appear in the text. Forty-six chromosomes are present in all except figure 8, in which there are 47. All figures are from metaphases in leukocytes cultured from peripheral blood.

PLATE 140

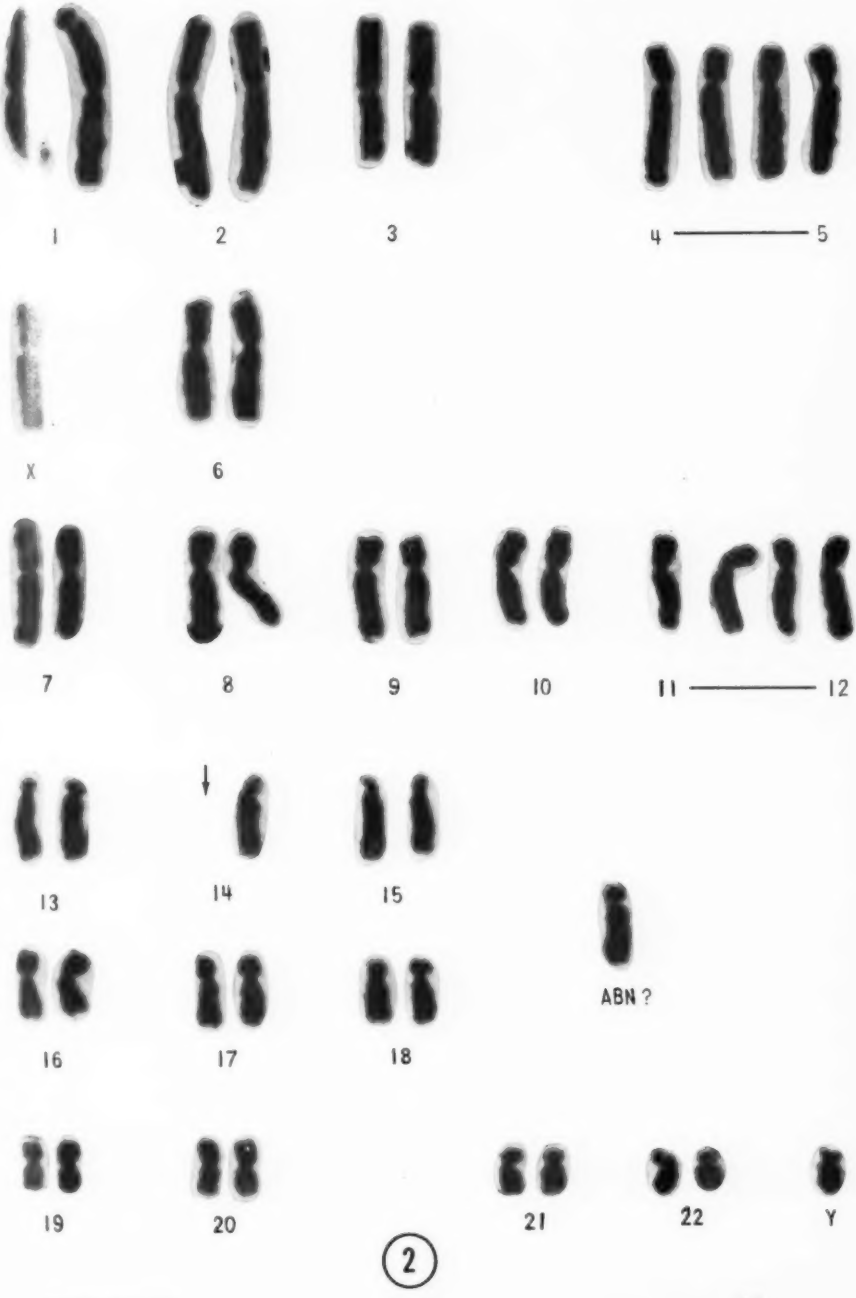
FIGURE 1.—Case 122T, a 3-month-old male. *Note* that one chromosome #6 is missing, and that an abnormal chromosome somewhat similar to #18 is present. One chromosome #16 is abnormally long. The X chromosome may be abnormal.



(1)

PLATE 141

FIGURE 2.—Case 124T, an 11-year-old male. *Note* that one chromosome #14 is missing, and that an apparently abnormal chromosome is present.



(2)

PLATE 142

FIGURE 3.—Case 124T. *Note* that the normal X and both chromosomes #6 are replaced by abnormal chromosomes.

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997

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PLATE 143

FIGURE 4.—Case 135T, a 6-year-old female. *Note* that 2 of the 4 group 11 to 12 chromosomes are replaced by abnormal chromosomes. Both chromosomes #10 may be abnormal.

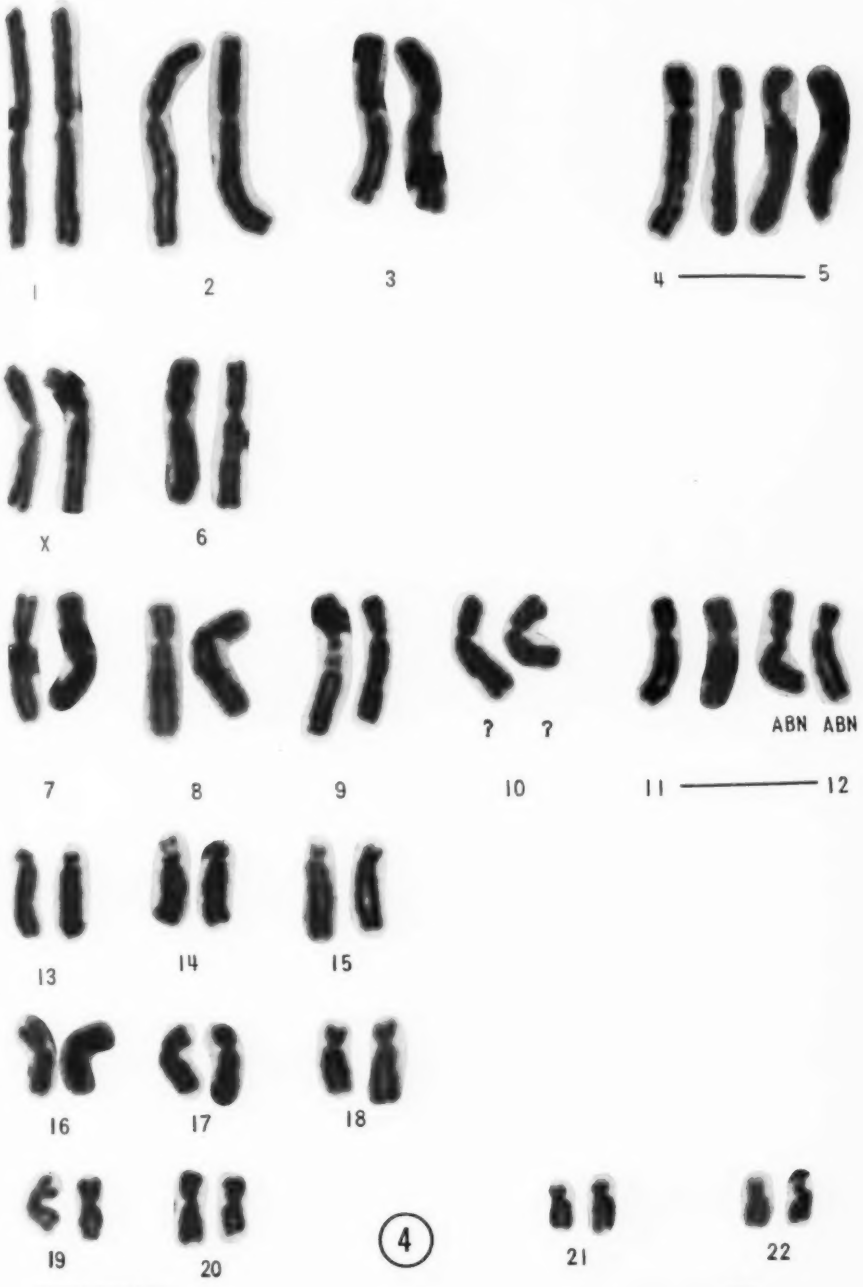

1
2
3
4
5
X
6
7
8
9
? ?
10
ABN ABN
11
12
13
14
15
16
17
18
19
20
4
21
22

PLATE 144

FIGURE 5.—Case 136T, a 3-year-old male. A total of 10 karyotype analyses was made, of which this is one. All were normal.

5

PLATE 145

FIGURE 6.—Case 137T, a 5-year-old male. *Note* absence of a normal X and presence of an abnormal chromosome.

6

PLATE 146

FIGURE 7.—Case 137T. *Note* that 1 chromosome #20 is missing and that an abnormal chromosome somewhat similar to 18 is present. *Note* also that 1 of the four 21 to 22 chromosomes has abnormally large satellites and stalks. One chromosome #6 may be abnormal.

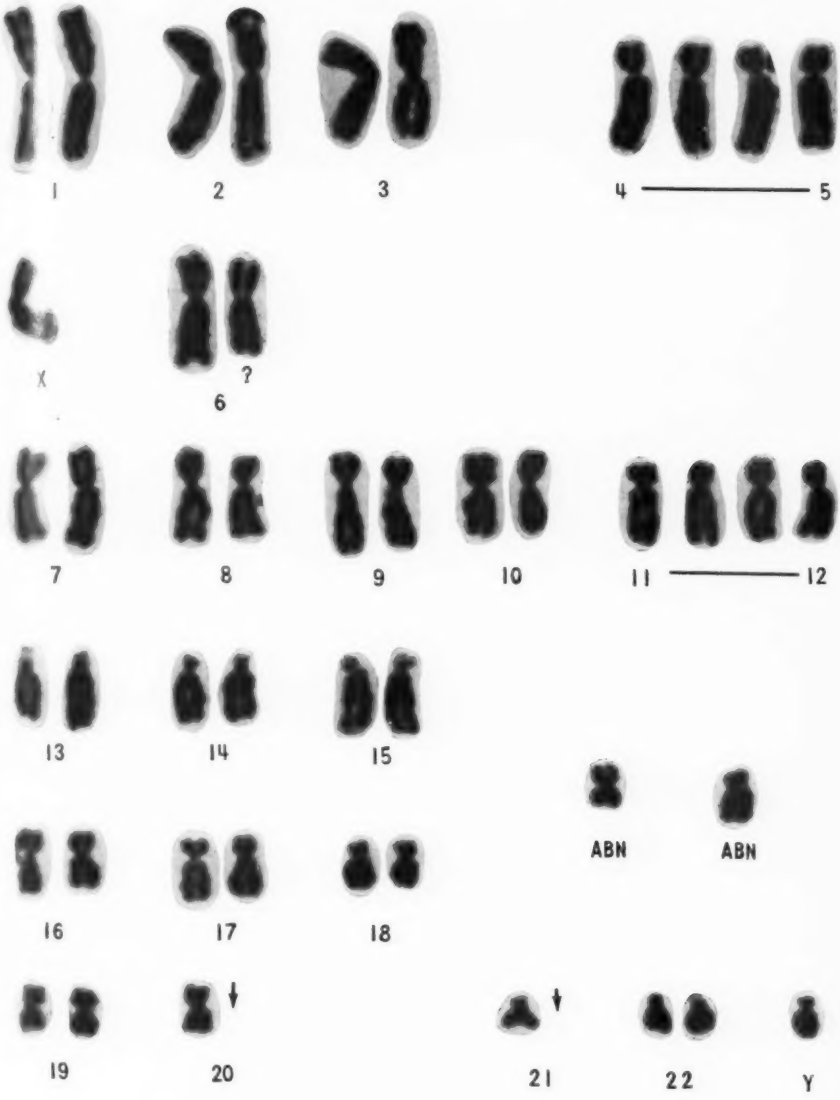
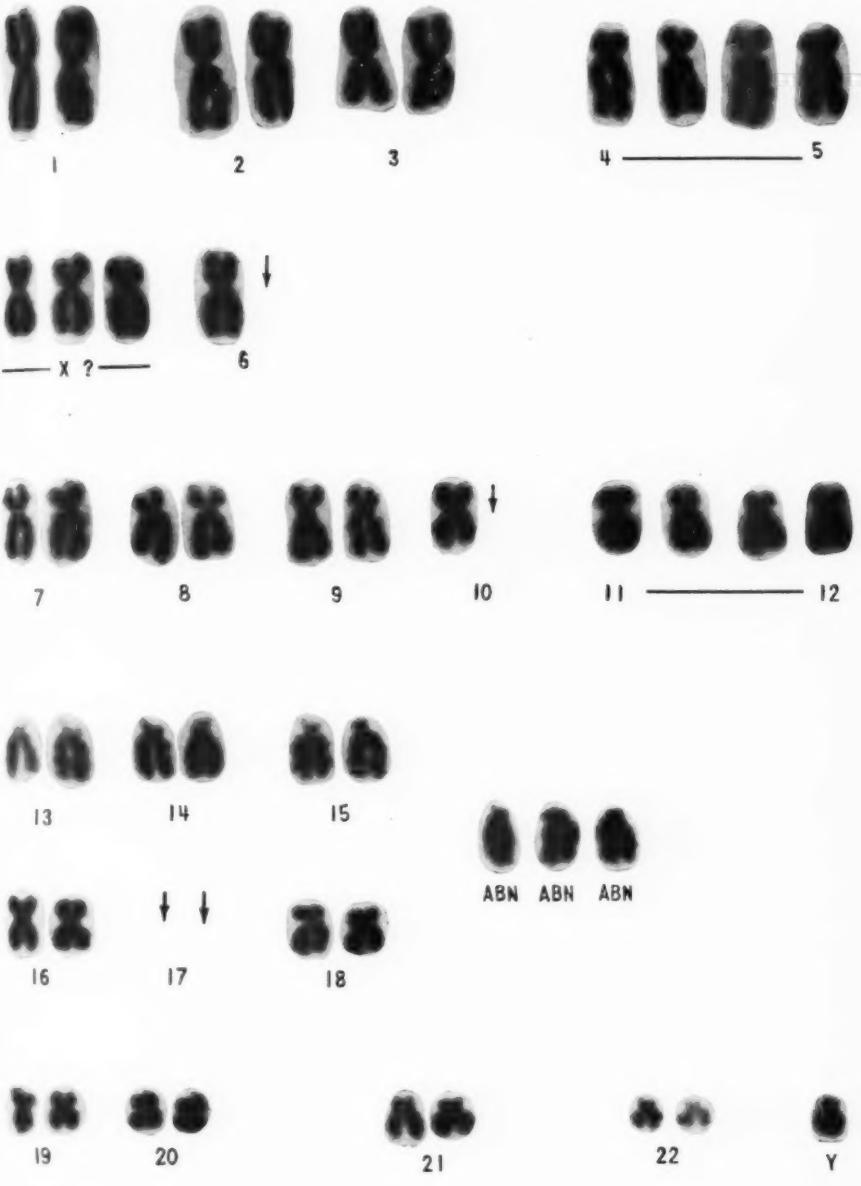

1
2
3
4
5
X
?
6
7
8
9
10
11
12
13
14
15
ABN
ABN
16
17
18
19
20
21
22
Y

PLATE 147

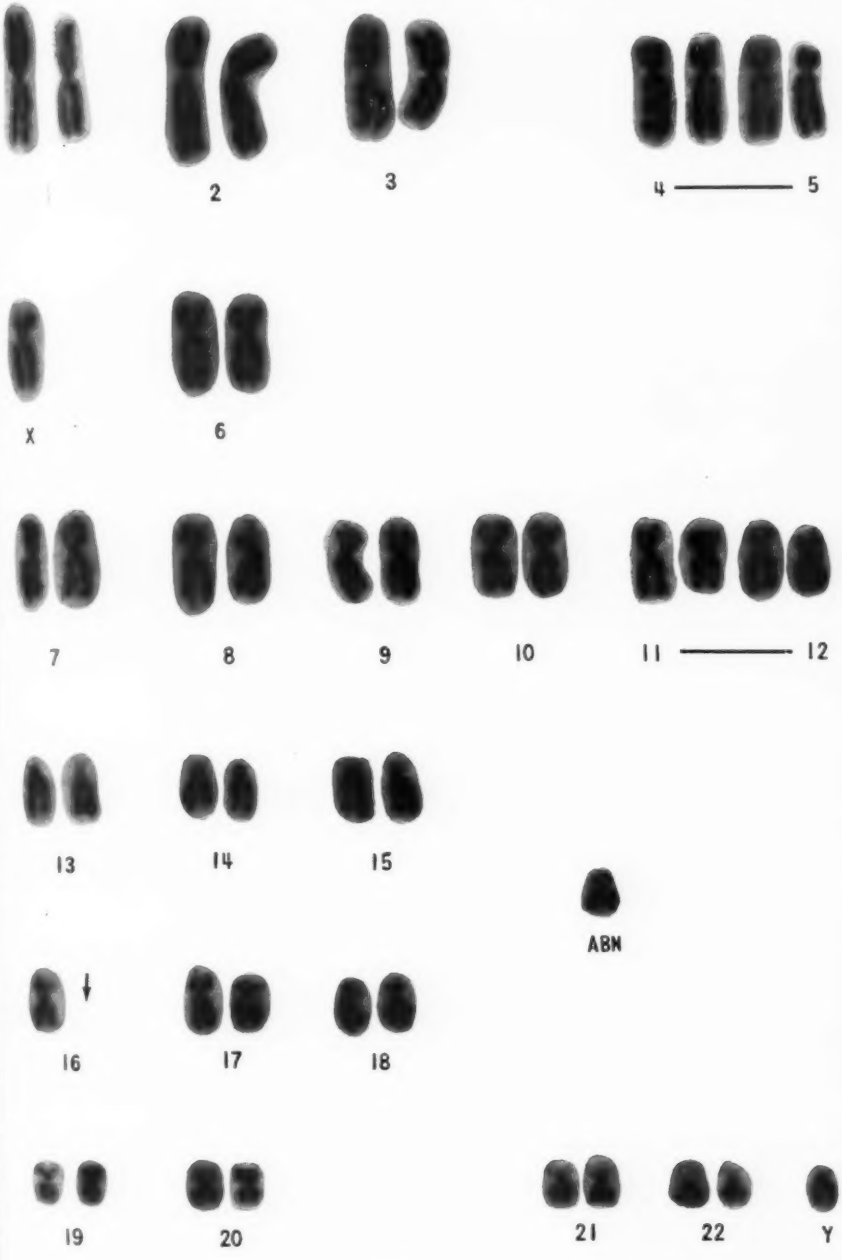
FIGURE 8.—Case 137T. *Note* that there are 47 chromosomes. One chromosome #6, one #10, and both #17's are missing. Three apparently normal chromosomes, identical with X, are present, and 3 abnormal chromosomes similar in morphology to group 13 to 15 are present.



8

PLATE 148

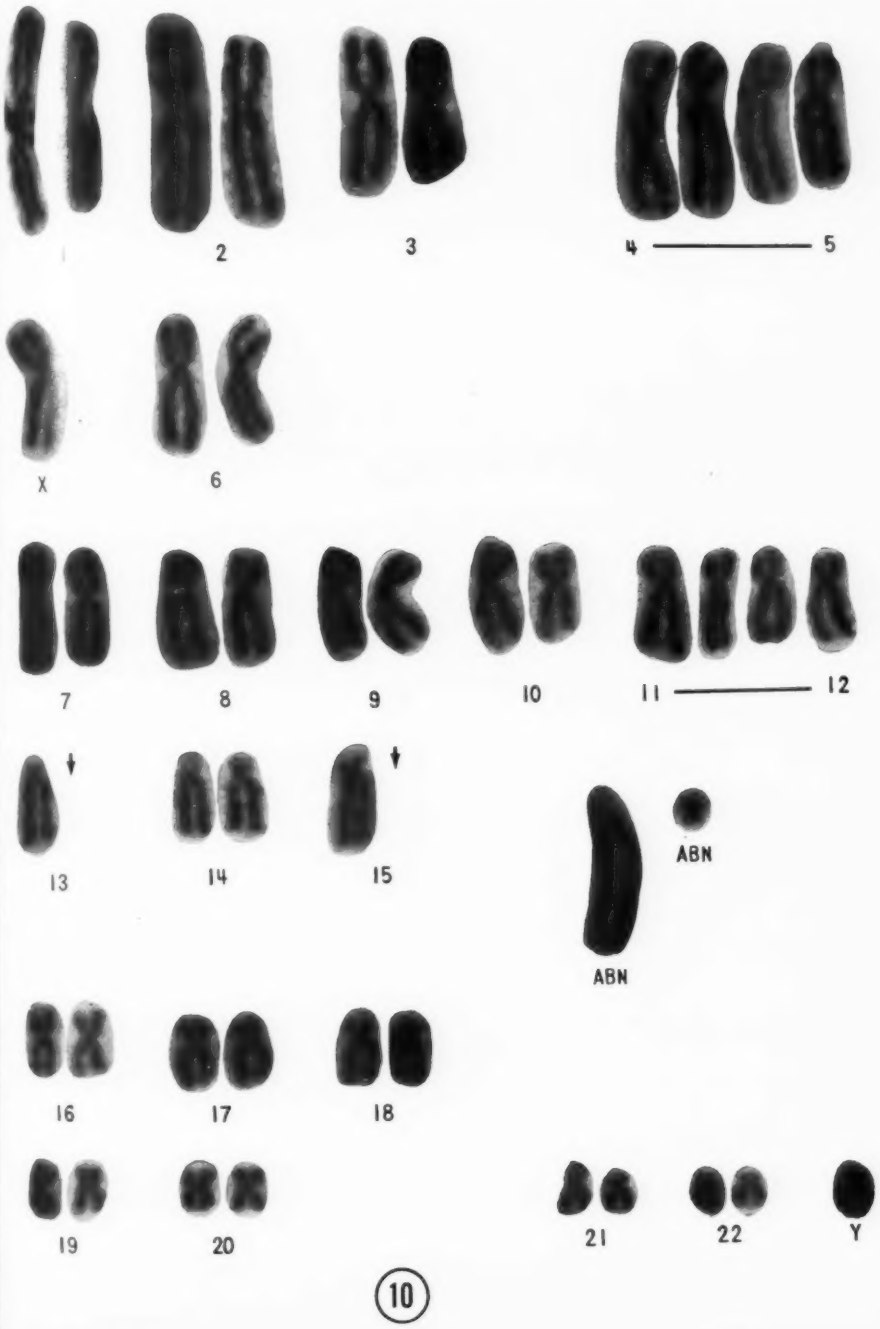
FIGURE 9.—Case 138T, a 5-year-old male. *Note* absence of 1 chromosome #16 and presence of an abnormal chromosome, similar to but larger than the group 21 to 22 chromosomes.



9

PLATE 149

FIGURE 10.—Case 146T, an 8-year-old male. Instead of 2 of the group 13 to 15 chromosomes, an abnormally long acrocentric and a minute are present.



(10)

Chromosome Studies in Human Leukemia. II. Chronic Granulocytic Leukemia [1]

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SUMMARY

Chromosome studies in 10 patients with chronic granulocytic leukemia are reported. The patients examined varied from asymptomatic untreated cases to extensively treated cases of several years' duration in terminal myeloblastic crisis. In 9 of the 10 patients, a similar chromosome abnormality was found. One of the four smallest acrocentric chromosomes in the leukemic cells was markedly reduced in size, apparently due to the loss of approximately half of its longer arm. With the exception of this finding, no frequent or regular abnormality in either chromosome number or structure was observed. The findings demonstrate a specific, consistent chromosome change associated with a particular human neoplasm and suggest that it is the chromosome change which confers on the leukocytes their neoplastic character. Such an association has not yet been demonstrated in any other human tumor.—J. Nat. Cancer Inst. 27: 1013–1035, 1961.

RECENTLY AN abnormally small chromosome was described in the leukemic cells of 2 patients with chronic granulocytic leukemia (*1*). Subsequently, the same abnormality was observed in additional cases studied in our laboratory (*2*) and by the group in Edinburgh (*3*, *4*), who have designated the abnormal chromosome Ph[1].

The present paper contains the chromosome findings in 10 patients, ranging in age from 9 to 80 years, with chronic granulocytic leukemia. These have not been reported previously in detail.

MATERIALS AND METHODS

Chromosome counts and karyotype analyses were made from short-term cultures (48 and 72 hours) of peripheral blood cells, and, in 2 cases,

[1] Received for publication June 27, 1961.

[2] Supported in part by senior research fellowship SF-4 from the Public Health Service and research grant C-4659 from the National Cancer Institute, National Institutes of Health, Public Health Service.

[3] Supported in part by a terminal year predoctoral research fellowship, CF-11, 168, and in part by research grant C-1613, from the National Cancer Institute, National Institutes of Health, Public Health Service.

[4] The authors wish to express their appreciation to Dr. M. Goldwein, Dr. J. Frost, Dr. A. J. Donnelly, Dr. M. Sachs, Dr. S. Skromak, Dr. A. Weiss, and Dr. I. Wolman, who made clinical material available for this study, and to Miss S. Daugherty, Miss S. Ferry, Mrs. E. Krohnert, and Miss J. Wagner for able technical assistance.

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from direct preparations or 24-hour cultures of bone marrow. The techniques employed have been described in detail elsewhere (*5*, *6*).

CASE HISTORIES

125T.—E.C., a 41-year-old female was hospitalized April 3, 1960, because of weakness and splenomegaly of 1 month's duration. The white blood count was 450,000; bone marrow diagnosis: "chronic granulocytic leukemia." She was treated with Myleran and achieved a partial remission which continues to date. Blood samples were obtained for chromosome study April 6, 1960 (before therapy was started), and September 1, 1960. White blood cell counts at these times were, respectively, 450,000 (blasts and promyelocytes, 5%; myelocytes, 34%; metamyelocytes, 32%; bands and segmenters, 25%; eosinophils, 2%; lymphocytes, 2%) and 22,000 (blasts and promyelocytes, 10%; myelocytes, 3%; metamyelocytes, 11%; bands and segmenters, 66%; eosinophils, 2%; basophils, 4%; lymphocytes, 4%).

133T.—M.W., a 65-year-old white female, was hospitalized June 14, 1960, for treatment of an aneurysm of the brachial artery. She had no symptoms suggesting a hematological disorder. A routine white blood cell count revealed a marked elevation, and a bone marrow examination confirmed the diagnosis of chronic granulocytic leukemia. Treatment with Myleran produced a hematological remission which continues to date. Samples of blood and bone marrow were obtained June 16, 1960, before therapy was started. At that time the white blood count was 51,000 (blasts and promyelocytes, 6%; myelocytes, 10%; metamyelocytes, 11%; bands and segmenters, 67%; eosinophils, 3%; lymphocytes, 3%).

139T.—W.M., a 28-year-old Negro male, whose case was first diagnosed as "acute granulocytic leukemia" in September, 1959, was treated with 6-mercaptopurine and steroids and attained a remission which lasted until December, 1959. At that time, he was hospitalized with a leukocyte count of 62,000 and a differential count typical of chronic granulocytic leukemia. He was treated intermittently over the next 10 months with Cytotoxan, 6-mercaptopurine, and steroids. During this period his leukocyte count fluctuated widely between 3,000 and 300,000, and there was considerable doubt as to the regularity with which he was taking his medication. His differential count remained typical of chronic granulocytic leukemia, platelet counts remained elevated, and anemia was not marked. In October and November, 1960, his white count gradually increased, and anemia and thrombocytopenia developed. He died suddenly of cerebral hemorrhage in November, 1960. Blood samples for chromosome study were obtained August 2, 1960, and October 18, 1960. White blood cell counts at these times were, respectively: 12,800 (myelocytes, 4%; metamyelocytes, 10%; bands and segmenters, 58%; eosinophils, 1%; basophils, 8%; lymphocytes, 16%; monocytes, 3%) and 202,000 (blasts and promyelocytes, 12%; myelocytes, 13%; metamyelocytes, 32%; bands and segmenters, 33%; eosinophils, 2%; basophils, 2%; lymphocytes, 4%; monocytes, 2%).

142T.—S.G., a 32-year-old white male, whose case was diagnosed as chronic granulocytic leukemia in 1955, was treated with P^{32} and attained a remission which lasted, on maintenance therapy with P^{32}, until July, 1960, when an acute exacerbation of the leukemia occurred, which was treated with 6-mercaptopurine, with only partial success. A second exacerbation, in September, 1960, failed to respond to therapy and he died September 30, 1960. Blood for chromosome study was obtained September 6, 1960; at that time the white blood count was 27,000 (blasts and promyelocytes, 58%; myelocytes, 13%; metamyelocytes, 12%; bands and segmenters, 8%; eosinophils, 1%; basophils, 4%; lymphocytes, 4%).

144T.—E.R., a 36-year-old-white female, whose case was diagnosed as chronic granulocytic leukemia in June, 1958, was treated with Myleran until December, 1958,

and attained a remission which lasted until September, 1960. She was hospitalized with an acute exacerbation September 4, 1960, and was treated with 6-mercaptopurine which produced a temporary remission. She suffered a relapse in November, 1960, and died of her disease in December, 1960. Several blood and marrow samples were obtained between September 7 and December 20, 1960. Only 1 culture, however, grew sufficiently well to provide material for chromosome study. This specimen was obtained September 19 when the patient was entering a brief remission, and her circulating leukocytes, for a few days, were almost exclusively micromyeloblasts. At this time her white blood count was 2,500 (micromyeloblasts, 90%; bands and segmenters, 6%; lymphocytes, 4%).

149T.—R.E., an 80-year-old white female, developed typical chronic granulocytic leukemia (WBC = 800,000) in February, 1958. In 1956, she had received extensive X-ray therapy to the neck (5000 r) for inoperable, anaplastic carcinoma of the thyroid, with resultant arrest of her tumor. She was treated with Myleran until May, 1958, and attained a remission lasting until October, 1960. Mild exacerbations since that time have been successfully controlled with Myleran. A blood sample for chromosome study was obtained January 18, 1961. At that time the white blood count was 52,000 (promyelocytes, 8%; myelocytes, 11%; metamyelocytes, 13%; bands and segmenters, 51%; eosinophils, 1%; basophils, 8%; lymphocytes, 7%; monocytes, 1%).

150T.—J.B., a 34-year-old white male, whose case was diagnosed as chronic granulocytic leukemia in October, 1959, was treated with Myleran and steroids until September, 1960, without attaining complete remission. He was hospitalized at that time because of severe anemia, and the Myleran therapy was stopped. After transfusions, he was discharged and treated with steroids alone. He relapsed and died of his disease in March, 1961. A blood sample was obtained on October 7, 1960; at that time the leukocyte count was 22,500 (blasts and promyelocytes, 10%; myelocytes, 34%; metamyelocytes, 1%; bands and segmenters, 18%; eosinophils, 1%; basophils, 9%; lymphocytes, 26%; monocytes, 1%).

163T.—R.D., a 34-year-old white male, was hospitalized on January 18, 1961, with a history of left chest pain and weakness of 1 month's duration. Examination of blood and bone marrow revealed typical chronic granulocytic leukemia. Therapy with Myleran was started January 19, 1961, and produced a partial remission which has lasted to date. A blood sample was obtained January 23, 1961. At that time the white blood count was 258,000 (promyelocytes, 4%; myelocytes, 16%; metamyelocytes, 23%; bands and segmenters, 53%; eosinophils, 3%; lymphocytes, 1%).

168T.—S.S., a 9-year-old white female, whose case was diagnosed as chronic granulocytic leukemia (WBC = 51,000; differential count and bone marrow typical of chronic granulocytic leukemia), in December, 1960, was treated with Myleran for 1 month and achieved a nearly complete remission which continues to date. From the age of 2 years until the onset of her leukemia, this child had *annually* been subjected to a series of diagnostic X rays including several upper gastrointestinal series, barium enemas, and intravenous urograms. The total whole-body X-ray dose over this period was estimated to be approximately 50 r. Blood and bone marrow specimens for chromosome study were obtained on February 16, 1961. At that time, the white blood count was 11,000 (myelocytes, 3%; metamyelocytes, 5%; bands and segmenters, 49%; eosinophils, 6%; basophils, 2%; monocytes, 1%; lymphocytes, 34%). The bone marrow was hematologically normal.

170T.—P.H., a 10-year-old colored female, was first studied in 1956, at age 5, when blood and bone marrow findings were typical of chronic granulocytic leukemia. Her disease followed a very benign course, requiring only occasional X-ray therapy to the spleen for adequate control. In late February, 1961, an acute exacerbation occurred and she required hospitalization. A blood sample was obtained on February 27, 1961. At that time she had received no X-ray therapy or medication for over a year; her leukocyte count was 92,800 (blasts and promyelocytes, 10%; myelocytes, 6%; metamyelocytes, 13%; bands and segmenters, 64%; eosinophils, 2%; basophils, 3%; lymphocytes, 2%).

RESULTS

The cytological studies on all 10 patients are summarized in table 1. In all patients in which adequate preparations were obtained, the modal chromosome number was consistently 46, and in all but 1 the characteristic abnormal chromosome, Ph^1, was observed (figs. 1 through 8 and 11 through 14). In 2 cases (149T and 163T), low mitotic index and poor quality of preparations made accurate characterization impossible. However, in both, the Ph^1 chromosome could definitely be recognized in many cells (figs. 7 and 8). As previously suggested (*4*) and as discussed below, it appears to be a chromosome 21 which has lost a portion of its longer arm.

The single patient in which the Ph^1 chromosome was not observed (144T) was entering a brief remission after an acute myeloblastic crisis at the time a blood specimen was obtained. Her white blood count was 2,500 with 90 percent of the cells identified as "micromyeloblasts." In some of her cells, 1 of the 4 smallest autosomes was slightly smaller than its homologue (figs. 9 and 10).

Metaphases were also found in case 144T in which all the small acrocentric autosomes were completely normal. Since the chromosome change appears to involve a chromosome 22 rather than a 21, its relationship, if any, to chronic granulocytic leukemia is not clear.

The only consistent chromosome abnormality other than the Ph^1 chromosome seen in any of the cases was a second similar abnormally small chromosome, also derived from 1 of the 4 smallest acrocentric autosomes, observed in some of the cells in 142T (figs. 11 and 12). This individual had the longest history of any of the patients examined (5 years) and throughout this period had been treated with P^{32}. This additional chromosome change is probably the result of extensive radiation therapy.

In addition to the data on case 150T in table 1, a further interesting observation was made. In the admixture on the same slides of cells with and without the Ph^1 chromosome, the chromosomes of cells without it had the appearance of being well fixed, while those with the abnormality invariably gave the impression of having been poorly fixed (figs. 13 and 14). The patient, having just completed a course of combined therapy with steroids and Myleran, was at this time on steroids. It is possible that this observed difference is the result of a differential response of the cells, either to the therapeutic agents used *in vivo* or to the conditions to which the cells were exposed *in vitro*. In 2 of the 7 cells from this patient which were subjected to karyotype analysis, 1 with and 1 without the Ph^1 chromosome, additional chromosome changes, apparently nonspecific, were noted. In the first cell, a #16 chromosome was missing and an abnormal chromosome was present. In the second cell, 1 arm of a group 19 to 20 chromosome was missing.

In most of the patients, some of the dividing cells showed a normal karyotype. These cells undoubtedly represent nonleukemic leukocytes.

TABLE 1.—Chromosome counts, karyotype analyses, and the Ph¹ chromosome in 10 patients with chronic granulocytic leukemia

Case number	Date sampled	Type of preparation	Counts					Total counts	Analyses*	Ph¹ chromosome
			43	44	45	46	47			
125T	4/6/60	Blood, 48-hour culture	2		1	6		24		Present
	9/1/60	Blood, 48- and 96-hour cultures			1	14			4	
133T	6/16/60	Marrow, direct				2		25	1	
		Marrow, 24-hour culture				17				Present
		Blood, 96-hour culture				5	1		2	
139T	8/2/60	Blood, 48-hour culture			2	13		56	4	Present
	10/18/60	Blood, 48-hour culture				10			1	Present
	10/18/60	Blood, 72-hour culture			2	29				Absent
142T	9/6/60	Blood, 72-hour culture	1		3	34		38	7	Present
144T	9/19/60	Blood, 48-hour culture			1	2		41		Absent
	9/19/60	Blood, 72-hour culture			1	37			7	Absent
149T	1/18/61	Blood, 72-hour culture						0	0	Present†
150T	10/7/60	Blood, 48-hour culture			2	21		23	7	Present
163T	1/23/61	Blood, 48-hour culture				1		1	1	Present†
168T	2/16/61	Marrow, 24-hour culture		1	1	8		10	3	Present†
170T	2/27/61	Blood, 48-hour culture			1	9		10	2	Present†

*See text for details of analyses.

†The Ph¹ chromosome was observed in several cells which could not be accurately counted or analyzed.

Since such cells rarely divide before 72 hours *in vitro*, they are observed infrequently in cultures of leukemic blood terminated at 48 hours. Conversely, dividing leukemic cells tend to be less frequent in 72-hour than in 48-hour cultures. For this reason leukemic cultures were generally terminated at 48 hours. In case 139T, both 48- and 72-hour cultures were grown from the blood sample obtained in October, 1960 (*see* table 1). The Ph^1 chromosome was observed only in the 48-hour cultures, and *not* in the 72-hour. In this instance, if only 72-hour cultures had been examined, case 139T would have been incorrectly recorded as negative for the Ph^1 chromosome.

In addition, as would be expected, cells with normal chromosome complements are apparently less frequent in the leukemic cultures containing a very high percentage of young myeloid forms. It would be of interest to determine whether there is a consistent proportional relationship between the frequency of the Ph^1 chromosome and of immature myeloid cells in the inoculum.

DISCUSSION

The identification of the normal chromosome from which the Ph^1 chromosome is derived presents some difficulty. Apparently, it is one of the 4 group 21 to 22 chromosomes, but the degree of certainty with which the discrimination can be made varies, to a large extent, with the degree of metaphase chromosome contraction in a given cell. To make such a distinction, the presence of satellites is not useful since they have been observed on all 4 of these chromosomes (fig. 1). However, in the best metaphases 2 of the 4 chromosomes are distinctly longer, this difference largely due to somewhat longer short arms. On this basis, the Ph^1 chromosome is regarded as having been derived from a chromosome 21.

Whether the mechanism for this change is translocation or deletion cannot yet be said. It may be that the deletion of such a large amount of autosomal chromatin would be lethal for the cell. If a translocation is responsible, the reciprocal product has not been identified. This is not entirely unexpected, since this amount of chromatin attached to a long arm of one of the larger chromosomes would be extremely difficult to recognize. We are in general agreement with the Edinburgh group (*4*) on these points.

While the morphology of the altered chromosome is remarkably similar from case to case, involving as it does the loss of approximately one half the long arm of a normal chromosome, it appears that there may be slight differences among these chromosomes in the different individual cases. The designation Ph^1 has undoubted value as a generic term, but its use should not be taken to imply that this chromosome is necessarily identical in every case. The relative crudeness of present-day metaphase chromosome analysis in humans makes such a determination impossible. We have adopted the term Ph^1 with this reservation in mind.

It appears possible that the chromosome abnormality observed in

chronic granulocytic leukemia may be a primary change rather than a secondary phenomenon appearing during the course of the disease. It was observed in one of our patients (133T) at the time of diagnosis, prior to any therapy and before she developed any symptoms referable to the hematopoietic system. In several other cases the Ph^1 chromosome was observed in cells examined within a few days of the initial diagnosis.

The apparent presence of this chromosome change from the outset of the disease, plus the consistent finding of the same abnormal chromosome in association with this one particular form of leukemia, strongly suggests that it is this change in the genetic apparatus which confers on these cells their neoplastic character.

This observation sets chronic granulocytic leukemia apart from other varieties of leukemia. Cytological studies in our laboratories and elsewhere [*see* review by Hungerford (*6*)] indicate that none of the other common forms of leukemia—acute granulocytic, acute and chronic lymphocytic—is characterized by any specific chromosome abnormality demonstrable by present methods. Many of the inconstant changes in chromosome number and morphology, which have been observed, apparently represent secondary alterations unrelated to the initiation of the disease. A possible exception may be leukemia associated with mongolism. The excess of genetic material of chromosome 21 in the cells of mongoloid children may be causally related to the increased incidence of leukemia correlated with this syndrome (*4*).

Chromosome studies may aid in the evaluation of certain current theories of leukemogenesis. The finding of a consistent chromosome abnormality in chronic granulocytic leukemia would seem to necessitate modification of the recent suggestion (*7*) that this disease is initiated by a deficiency in the patient of some circulating inhibitor of cell division, which permits uncontrolled multiplication of genetically normal myeloid cells. In this regard, it is of interest to note that in our cultures, the cells of chronic granulocytic leukemia generally grow better in normal (homologous) plasma, which should contain the hypothetical inhibitor, than they do in leukemic (autologous) plasma, which should be deficient.

The gross chromosome aberration observed in the cells of chronic granulocytic leukemia is of a type known to be produced by ionizing radiation, and 2 of the patients in the present series (149T and 168T) had been exposed to large doses of X ray prior to the onset of leukemia. It is possible that chronic granulocytic leukemia results from the effects of ionizing radiation or other mutagenic agents which produce chromosome breakage. In the well-documented instances of radiation-induced human leukemia (*8*, *9*), however, acute leukemias have been as frequent as the chronic granulocytic form, and no specific chromosome change has yet been demonstrated in acute leukemia (*6*). These latter cases might, of course, be the result of radiation-induced point mutation(s) which would not be demonstrable by present cytological techniques.

The question of the consistency of the chromosome change in chronic granulocytic leukemia should also be considered. Of the 12 patients

whom we have examined to date [10 in this paper; 2 previously reported (*1*)], the Ph¹ chromosome has been absent in only one instance. A satisfactory specimen was obtained from this patient (144T) only when she was in an acute crisis and her peripheral leukocytes were almost exclusively "micromyeloblasts." In the 18 cases reported by the group in Edinburgh (*4*), the Ph¹ chromosome was not observed in 5. Three of these "negative" cases had very few immature myeloid cells in the peripheral blood specimens which were cultured. Such cases should probably not be considered negative until bone marrow has also been examined for the Ph¹ chromosome. Our case 168T was of this type. The patient was in clinical and hematological remission. The blood specimen that was cultured contained very few myeloid cells capable of division (blasts, promyelocytes, and myelocytes) and the Ph¹ chromosome was not observed. However, a high percentage of marrow cells, cultured at the same time, contained the Ph¹ chromosome. The other 2 negative cases reported by the Edinburgh group were in acute "blast" crises at the time of culture, as was our case 144T. The significance of this observation must await further investigation, particularly as to whether patients whose cells have contained the Ph¹ chromosome during typical early stages of chronic granulocytic leukemia may fail to show it during subsequent acute exacerbations.

It is also of interest that, in our case 144T, the alkaline phosphatase level in the patient's circulating granulocytes was apparently not reduced. Although there were only a few mature granulocytes in the specimen obtained during her terminal blast crisis, all the 12 cells examined were scored as "2+" or "3+". This concentration of alkaline phosphatase is considerably greater than normal and is in sharp contrast to the much reduced phosphatase levels usually found in this disease (*10*). It seems possible that the chromosome change in the cells of chronic granulocytic leukemia is of importance in influencing not only their proliferative capacity but also their level of alkaline phosphatase. The fact that leukocyte phosphatase levels were apparently not reduced in case 144T may be related to the observation that in this patient there is no visible change in chromosome 21, though there appears to be a slight change in 1 chromosome 22 in some of the metaphases. However, in this case, both the chromosome studies and the determinations of phosphatase levels were made during an atypical stage of the disease.

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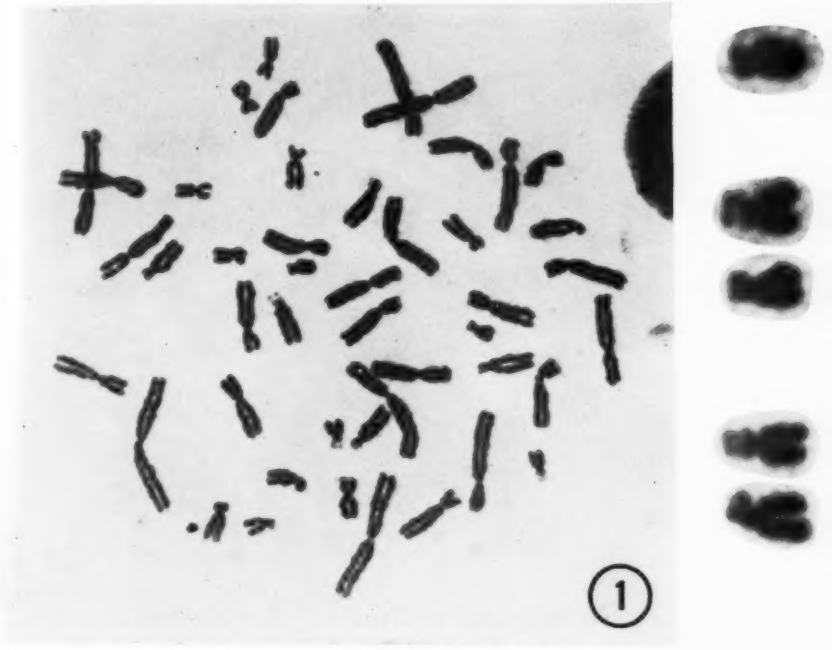
Metaphase plates are magnified approximately 1,800 diameters; chromosomes shown at *right* of figures 1, 2, 3, 9, 10, 11, and 12 are magnified approximately 5,000 diameters.

PLATE 150

FIGURE 1.—Normal metaphase from peripheral blood culture of case 139T (male, 28 years). Chromosomes at *right* are, from bottom to top, 21, 21, 22, 22, and Y. *Note* that all 4 of group 21 to 22 autosomes are satellited.

FIGURE 2.—Leukemic metaphase from case 139T. *Note* the Ph^1 chromosome (*arrow*). To *right* are shown, from bottom to top, 21, Ph^1, 22, 22, and Y. The Ph^1 chromosome is apparently a 21 which has lost approximately one half of its long arm.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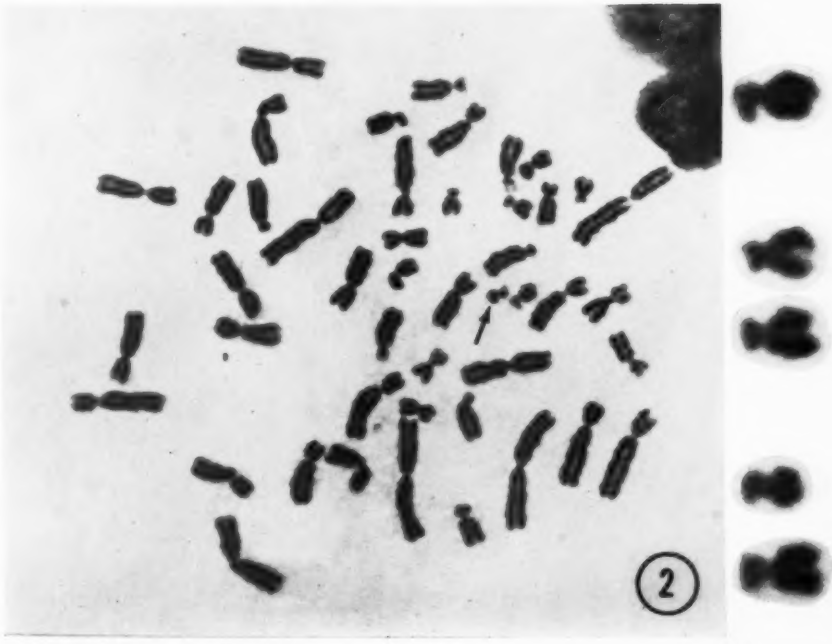
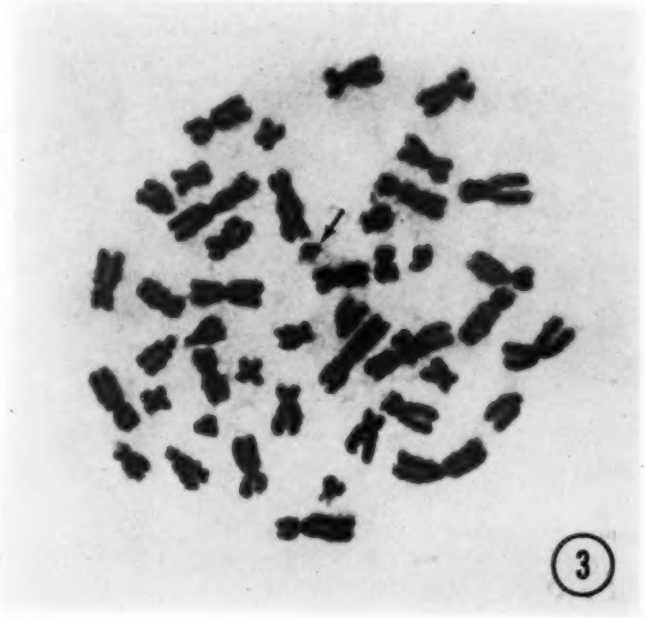

2

PLATE 151

FIGURE 3.—Ph^1 chromosome (*arrow*) in a metaphase from peripheral blood culture of case 125T (female, 41 years). To *right* are shown, from *bottom* to *top*, 21, Ph^1, 22, and 22.

FIGURE 4.—Ph^1 chromosome (*arrow*) in a direct bone marrow preparation from case 133T (female, 65 years). Patient was asymptomatic. A routine blood count was abnormal, and the original diagnosis of chronic granulocytic leukemia was made on the same marrow specimen from which this chromosome preparation was obtained.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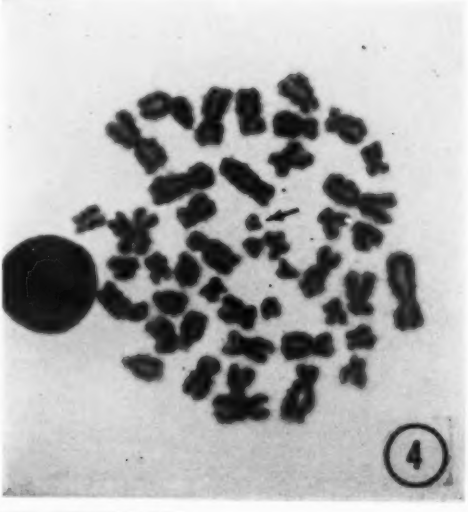
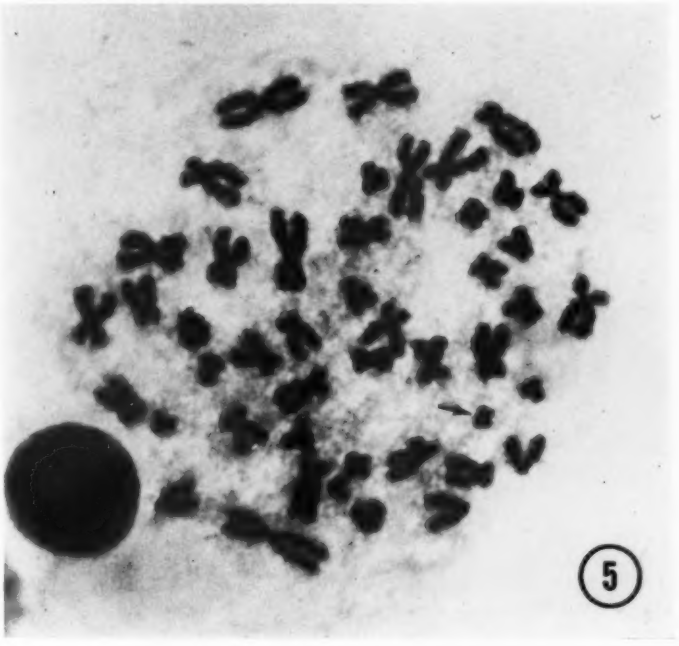

4

PLATE 152

FIGURE 5.—Ph[1] chromosome (*arrow*) in a metaphase from 24-hour marrow culture of case 168T (female, 9 years). This child was in clinical and hematological remission when studied.

FIGURE 6.—Ph[1] chromosome (*arrow*) in a metaphase from peripheral blood culture of case 170T (female, 10 years). This child had had chronic granulocytic leukemia since the age of 5 years.

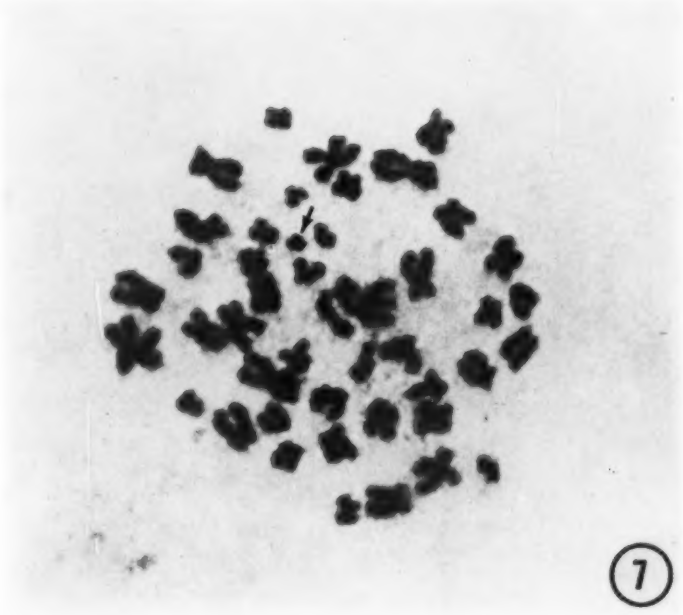

5

6

PLATE 153

FIGURE 7.—Ph^1 chromosome (*arrow*) in a metaphase from peripheral blood culture of case 149T (female, 80 years). In this case, and in case 163T, accurate counts were not possible, but the Ph^1 chromosome was easily recognized in many cells.

FIGURE 8.—Ph^1 chromosome (*arrow*) in a metaphase from peripheral blood culture of case 163T (male, 34 years).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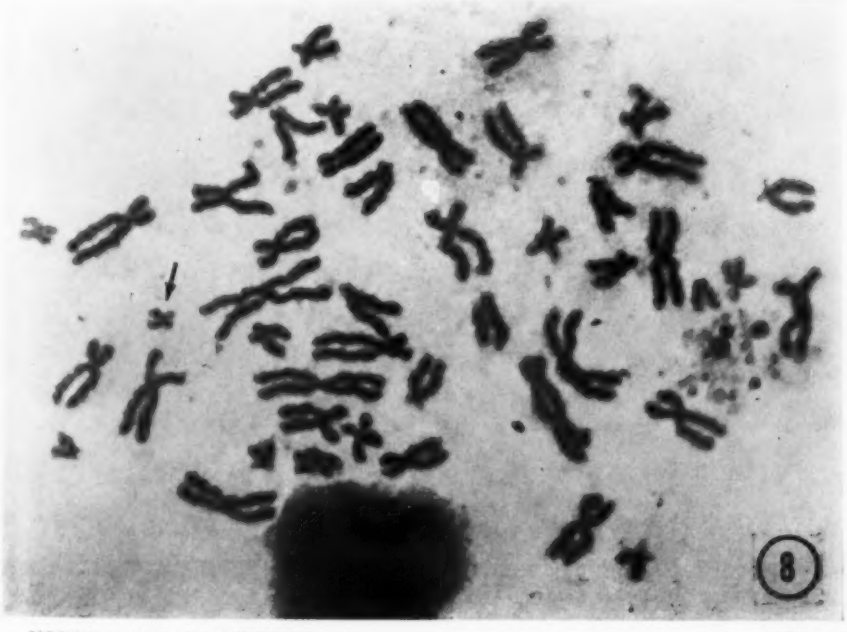
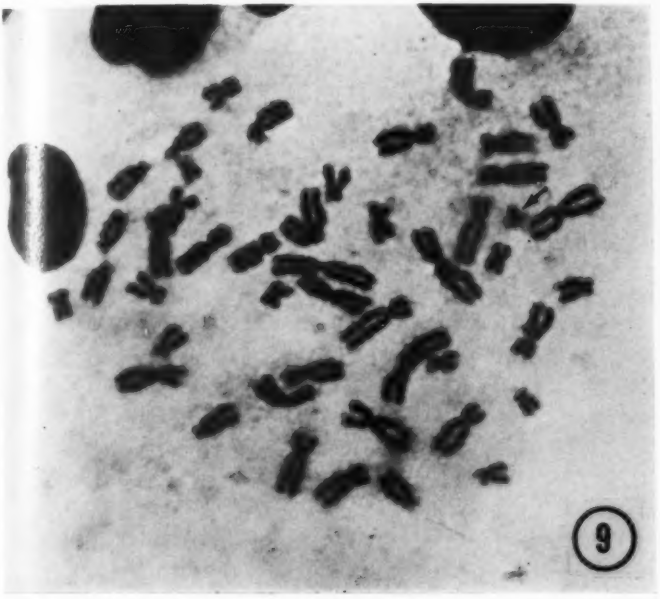

8

PLATE 154

FIGURE 9.—Metaphase from peripheral blood culture of case 144T (female, 36 years), the only case in which the Ph^1 chromosome was absent. *Arrow* indicates a somewhat atypical acrocentric autosome. This change was observed in a number of cells. Its relationship to the disease, if any, is not clear. It is shown *uppermost* in group to *right*, and is apparently derived from a chromosome 22.

FIGURE 10.—A metaphase from peripheral blood of case 144T. Atypical chromosome shown in figure 9 is apparently not present.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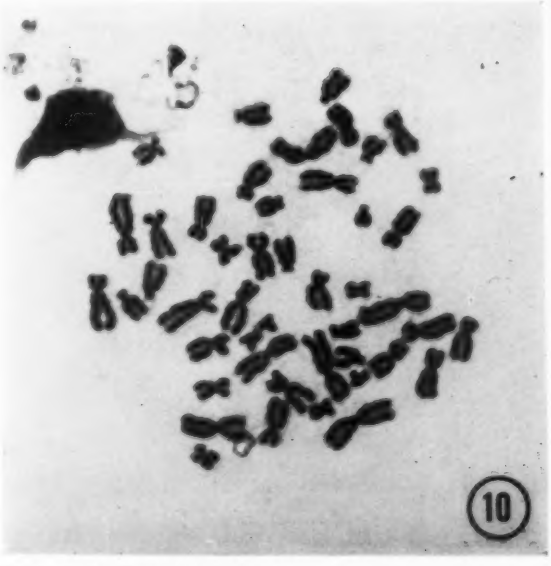
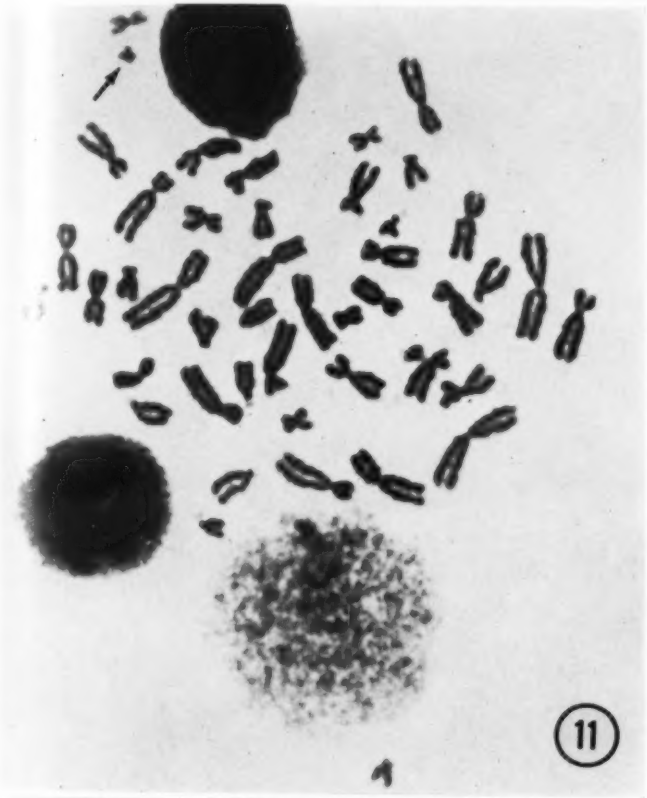
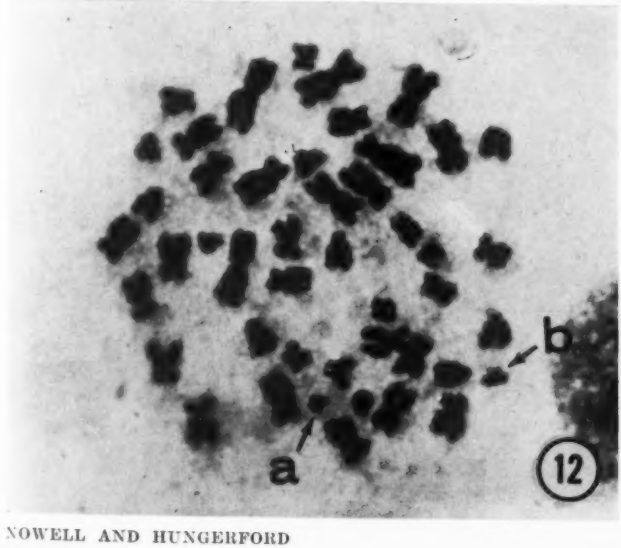

10

PLATE 155

FIGURE 11.—Ph[1] chromosome (*arrow*) in a metaphase from peripheral blood culture of case 142T (male, 32 years) Chromosomes at *right* are from *bottom* to *top*, 21, Ph[1], 22, 22, and Y.

FIGURE 12.—Metaphase from same culture as figure 11 with typical Ph[1] chromosome (*arrow, a*) and a second, similar, abnormally small chromosome (*arrow, b*), present in place of 1 normal chromosome 22. This was seen in many cells and is the only consistent abnormality other than the Ph[1] chromosome observed in any of the cases. *See* text for further discussion of this observation. Chromosomes at *right* are, from *bottom* to *top*, 21, Ph[1], 22, second abnormal chromosome, and Y.


11


a
b
12

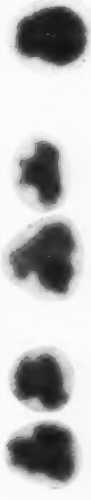
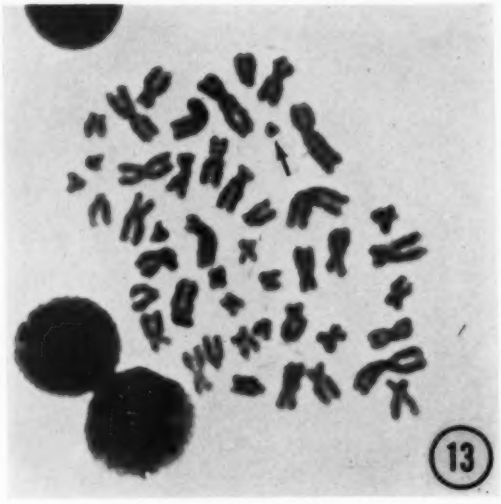


PLATE 156

FIGURE 13.—The Ph^1 chromosome (*arrow*) in a metaphase from peripheral blood culture of case 150T (male, 34 years). *Note* appearance of poor fixation characteristic of *all* cells bearing the Ph^1 chromosome (*i.e.*, leukemic cells) in this culture.

FIGURE 14.—Normal metaphase from same culture as figure 13. *Note* appearance of better fixation characteristic of cells *not* bearing the Ph^1 chromosome (*i.e.*, non-leukemic cells).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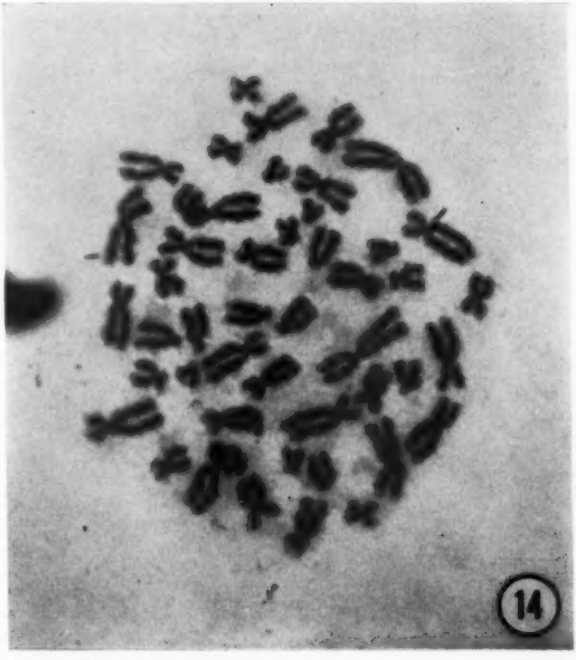
14

Spontaneous Tumors in the Sprague-Dawley Rat [1]

S. W. THOMPSON, *D.V.M., M.S.*, R. A. HUSEBY, [2] *M.D., Ph.D.*, [2] MARY A. FOX, *A.B.*, C. L. DAVIS, *D.V.M.*, [3] *and* R. D. HUNT, *D.V.M., Pathology Division, U.S. Army Medical Research and Nutrition Laboratory, Fitzsimons General Hospital, Denver, Colorado*

SUMMARY

The pathology of 62 spontaneous tumors in 52 of 125 albino Sprague-Dawley rats, used in an experiment to determine the effects of feeding a 100 percent irradiated diet, is described. The incidence of neoplasia in rats on irradiated diet and control animals showed no significant difference. The over-all incidence was 41.6 percent. Mammary tumors accounted for the largest single group, as in the reports of most investigators. A number of neoplasms were found and described, which to our knowledge have not heretofore been reported. These consist of a cavernous hemangioendothelioma of the oviduct, 2 fibrovascular polyps of the cervix, an adenocarcinoma of the small intestine, an ependymoma, a papilloma of the choroid plexus, and a pinealoma. Primary tumors of the bone, lung, liver, thymus, adipose tissue, and male genital organs, as reported by other investigators, were not observed in our series. The incidence of lymphoid and cutaneous neoplasia was very low compared with some reports in the literature.—J. Nat. Cancer Inst. 27: 1037–1057, 1961.

DURING RECENT studies on the evaluation of food preserved by irradiation, an unexpectedly high incidence of spontaneous tumors was observed in control and experimental rats of the Sprague-Dawley strain. An analysis of the total number as well as the incidence of tumors arising in the various organ systems showed no significant difference between the control and experimental animals [5% probability level, Read (*1*)]. Therefore, for the purpose of this paper, the neoplasms observed will be discussed as occurring in a single population, and reference will not be made to the control versus the experimental animals.

Review of the literature shows a marked strain difference as to the type and incidence of neoplasia as well as of non-neoplastic disease in rats. The importance of an investigator having knowledge of the spontaneous lesions and their incidence in his particular strain of rat need not be emphasized. Although the Sprague-Dawley strain cannot be considered

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inbred, there is little in the literature on the incidence of neoplasia in this widely used strain of rat except the article by Davis *et al.* (*2*) in which only 3 tumors of tissue other than the mammary gland are recorded. It is the purpose of this paper to discuss the occurrence of 62 tumors observed in 52 of 125 rats allowed to live out their lifespan.

MATERIALS AND METHODS

An original group of 34 female and 20 male albino rats received directly from Sprague-Dawley was started on the experiment, and they and their succeeding 3 generations were used to determine any effects of feeding a 100 percent irradiated diet. Studies on growth, reproductive performances, longevity, metabolizable energy, tissue enzyme activity, and histopathology were undertaken and are discussed by Read (*1*). The rats were housed individually in wire mesh cages with free access to fresh water.

Nine foods were used in the diet in the following dry weight percentages: bacon, 8.75; beef, ground, 8.43; haddock, 19.15; ham, fresh, 4.28; milk, powdered, 5.56; beets, 15.45; beans, green, 8.39; cereal, military, 3.00; peaches, commercial, frozen, 20.45. Hawk-Oser salt mixture, 3.00; Cellu-B, 1.00; gum acacia, 1.00; vitamin-methionine premix, 0.74; and choline-chloride, 0.26 were added to the diet. The 9 foods were irradiated at 5.58 megarad in a swimming-pool reactor (*1*). The irradiated foods were stored frozen until incorporated into the diets. The control animals were fed an identical diet that was nonirradiated and stored frozen. The approximate composition of the control diet in percent was: moisture, 77.72; protein, 6.90; fat, 3.68; ash, 1.36; carbohydrate (by difference), 10.34. The approximate composition of the irradiated diet in percent was: moisture, 77.63; protein, 6.88; fat, 3.74; ash, 1.34; carbohydrate (by difference), 10.41.

The first and second generations were allowed to continue on the experiment until they died or were killed when moribund. Their ages ranged from 7 to 32 months, with an average of 21 months. In cases of grossly visible tumors the animals were killed when the tumor impaired normal body movements or general health. No tumor transplantation experiments were performed. The third-generation animals were killed after completion of the breeding studies at an average age of 13 months. The first-, second-, and third-generation animals were used in breeding studies. Three rats of the first generation, 2 of the second, and 9 of the third did not have litters. The remaining female rats had an average of 2 litters each. The fourth generation was killed after 21 weeks on the experiment. Since the third- and fourth-generation animals were killed at an earlier age, a true incidence of spontaneous tumors can be determined only from the first two generations. Detailed necropsies were performed on all rats in this study. A thorough gross examination was made, and over 22 tissue blocks, representative of all organ systems regardless of

whether or not they appeared normal or abnormal, were taken for histopathological examination. Tissues were fixed in 10 percent neutral buffered formalin, paraffin embedded, and sections cut at 5 μ. Sections were stained with hematoxylin and eosin. A variety of special stains was used when indicated.

The anatomical terms used in this paper are taken from Greene's *Anatomy of the Rat* (*3*).

RESULTS

Of the 125 rats allowed to live their lifespan (first and second generation), there were 43 males and 82 females. When we tabulate the incidence of neoplasms, the occurrence of more than one neoplasm of identical type, such as mammary gland, in a single rat is considered as a single occurrence. In those animals in which more than one type of tumor was observed, each type is listed separately. A total of 62 tumors was observed in 52 rats; 10 rats had 2 primary tumors of different tissue origin. In addition, 6 rats had multiple mammary tumors, 5 with 2 and 1 with 3.

The incidence of tumor-bearing animals was 41.6 percent. This percentage compared with reports in the literature is of a moderate to high value. Crain (*4*) in his review of 786 rats of the Rochester strain of the Wistar rat had a tumor incidence of approximately 25 percent. Ratcliffe (*5*) in 2 colonies of rats (the Wistar stock colony and an experimental stock colony) had an incidence of 3.1 percent. Davis, Stevenson, and Busch (*2*) reported an incidence of 57 percent in the Sprague-Dawley rat. Bullock and Curtis (*6*) examined 31,868 rats from their own colony and reported an incidence of 1.53 percent. Saxton and co-workers (*7*) reported an incidence of 44.51 percent of spontaneous tumors in 498 albino rats of the Osborne-Mendel (Yale) strain.

All 62 of the neoplasms in the present study are listed in table 1. There was a total of 18 tumors in the males and 44 in the females. The greater incidence in the females can be accounted for by the larger number of female animals used in the experiment and the larger number of tumors involving the mammary gland and genital tract. Similar sex distribution has been described in other reports (*2*, *4*, *5*).

Tumors of the Mammary Gland

Both benign and malignant tumors occur spontaneously in the rat The benign lesions are much more common in the rat than in the mouse, where malignant tumors of mammary origin are common and benign tumors relatively rare. No viral factors have been demonstrated in the rat as in the mouse (*8*). Transplantability and continued growth are features of the benign mammary tumors of rats (*9–12*). The most common mammary tumor of the rat is the fibroepithelial tumor (fibroadenoma).

TABLE 1.—Summary of 62 spontaneous tumors occurring in 52 (16 males and 36 females) of 125 Sprague-Dawley rats allowed to live their lifespan *

Type of tissue and tumor	Female	Male
Mammary gland		
Fibroadenoma	12(E) 7(C)	—
Adenoma	1(C)	—
Adenocarcinoma	1(C)	—
Adrenal gland		
Pheochromocytoma	1(E) 1(C)	3(E) 2(C)
Cortical carcinoma	—	1(E)
Hypophysis		
Adenoma	2(E)	1(E) 2(C)
Thyroid		
Light-cell adenoma	4(E) 1(C)	3(E) 1(C)
Ovary		
Granulosa-cell tumor	1(C)	—
Oviduct		
Cavernous hemangioendothelioma	1(E)	—
Cervix		
Fibrovascular polyp	1(E) 1(C)	—
Uterus		
Leiomyosarcoma	1(E) 1(C)	—
Endometrial polyp	1(E)	—
Kidney		
Nephroblastoma	1(C)	—
Salivary gland		
Sarcoma	1(C)	—
Stomach		
Squamous-cell carcinoma	1(C)	—
Small intestine (ileum)		
Adenocarcinoma	—	1(C)
Pancreas		
Islet-cell adenoma	1(E)	—
Brain (and associated structures)		
Ependymoma	—	1(E)
Papilloma, choroid plexus	1(E)	—
Meningioma	1(E)	—
Pinealoma	—	1(C)
Skin and subcutaneous tissue		
Papilloma	—	1(E)
Fibrosarcoma	—	1(E)
Lymphoid organs		
Malignant lymphoma	1(C)	—
Total	44	18

*E = experimental; C = control.

Mammary tumors accounted for 21 or 47.7 percent of the 44 tumors observed in the female animals in this study and represented the largest group of neoplasms in this series. Nineteen fibroadenomas were observed

in female rats ranging in age from 11 to 30 months (21.78 average). One adenoma was observed in a 20-month-old rat, and 1 adenocarcinoma in a 22-month-old rat.

Benign tumors constitute the largest group in all reported studies (*2, 4–6, 9, 13–16*). Of the 32 mammary tumors reported by Dunning and Curtis (*9*), there were 2 adenomas, 2 adenolipomas, 5 fibromas, 21 fibroadenomas, 1 adenocarcinoma, and 1 fibrosarcoma. Bullock and Curtis (*6*) reported 94 tumors and noted 8 adenomas, 1 adenolipoma, 78 fibroadenomas, 2 carcinomas, 2 cystadenocarcinomas containing squamous epithelium, and 3 adenofibromas showing sarcomatous changes. Wright, Klinck, and Wolfe (*14*) described 149 mammary tumors of which there were 122 fibroepitheliomas, 10 fibromas, 11 adenomas, 5 cystadenomas, and 1 carcinoma. McCoy (*15*) reported in his survey of wild rats 54 subcutaneous tumors, all of which he classified as being benign. Davis, Stevenson, and Busch's survey (*2*) classed 65 of their 72 mammary tumors as benign: 46 adenofibromas, 18 fibromas, 1 adenoma, with 7 adenocarcinomas. Ratcliffe (*5*) reported 219 mammary tumors and classed 206 as benign: 2 fibromas, 1 lipoma, and 203 fibroadenomas, and 13 malignant neoplasms. Crain (*4*) in his series had 65 mammary neoplasms, 64 of which were benign: 53 fibroadenomas, 5 adenomas, 2 fibromas, 4 cystadenomas, and 1 carcinoma.

The highest recorded incidence of adenocarcinoma of the mammary gland is in the series reported by Davis *et al.* (*2*). Of 72 rats with mammary tumors, they classified 7 (10%) as adenocarcinomas.

The mammary tumors we observed were not unlike those reported by numerous investigators. No unusual features were noted in the tumors in our series.

Tumors of the Genital Organs

We did not find any tumors of the male genital system. The incidence of testicular tumors in rats is apparently variable. Davis and co-workers (*2*) did not report a single occurrence. Saxton *et al.* (*7*) found 1 testicular interstitial-cell tumor, and McCoy (*15*) reported 1 angiosarcoma. Bullock and Curtis (*6*) noted 1 testicular tumor, which they classed as a seminoma. Gilman and co-workers (*17*) observed 13 interstitial-cell tumors in 167 male rats. Testicular tumors accounted for a substantial portion of Crain's series (*4*) in which 35 of 200 tumors were classed as interstitial adenomas.

Ovary.—One rat had a 2.0 × 2.0 × 2.0 cm granulosa-cell tumor of the left ovary. This rat was killed at 22 months of age. Grossly, the tumor had both solid and cystic components. Histologically, the tumor mass consisted of 4 main lobes that were subdivided into lobules. Some of these were chiefly connective tissues with a smaller number of infiltrating granulosa cells; others were composed almost entirely of granulosa cells, with little orderly arrangement (fig. 1). Scattered throughout the mass were occasional fluid-filled, rounded, and open spaces suggesting abortive attempts to form graafian follicles. This rat had a bloody vaginal

discharge before death. The endometrium of the right uterine horn was hyperplastic, which suggested a possible excessive stimulation by estrogen. Iglesias (*18*) reported and successfully transplanted a granulosa-cell tumor in a rat and demonstrated estrogen production. Gilbert *et al.* (*19*) found a granulosa-cell tumor in a GG strain rat. Other types of ovarian tumors have been reported. Crain (*4*) in his series observed 2 fibromas and 1 cystadenocarcinoma. Bullock and Curtis (*6*) reported 3 cystadenocarcinomas, 1 fibroma, and 1 bilateral sarcoma. Saxton *et al.* (*7*) listed 5 adenomas and 2 fibromas. Ratcliffe (*5*) reported 3 carcinomas of the ovary.

Oviduct.—We observed 1 tumor of this structure, a cavernous hemangioendothelioma. It was seen grossly in a 20-month-old rat that died, and was thought to be an area of hemorrhage or possibly a postmortem change. However, on microscopic examination an area measuring 1.5 mm in the widest diameter was seen in which the lining epithelium was supported by a cavernous vascular submucosa that was composed of large, blood-filled spaces lined with endothelium (fig. 2). Tumors of the oviduct have heretofore not been reported in the rat. The only reports of a similar genital tumor are the endothelioma of the uterus described by McCoy (*15*) and a possible ovarian hemangioma reported by Kim, Clifton, and Furth (*11*).

Uterus.—Three of the 62 neoplasms we observed arose from the uterus. Two were classed as leiomyosarcomas and 1 an endometrial polyp. One of the leiomyosarcomas produced multiple serosal tumor implants throughout the peritoneal cavity. The primary tumor mass involved the lower half of the right horn and measured 8.0 × 3.0 × 3.0 cm. This tumor was observed in a 16-month-old rat that died. The other leiomyosarcoma and the endometrial polyp were in rats that were killed at 23 and 24 months of age, respectively.

Microscopically, the leiomyosarcomas were multilobulated and composed of a richly vascular, highly cellular tissue. The neoplastic cells varied in type within the tumor, from round and immature to oval-shaped (cigar) more mature-appearing cells. The general pattern was essentially whorled in more mature portions of the neoplasm. The less-differentiated areas of the mass had little tendency toward an organized pattern. There were focal hemorrhages within each neoplasm associated with irregularly shaped necrotic areas.

The endometrial polyp was from the left uterine horn and consisted of a proliferating tissue mass that projected into the lumen of the uterus. The mass was composed of connective tissue essentially the same as that of the normal endometrium. It was covered by epithelium on all free surfaces and contained subepithelial glands similar to those observed elsewhere in the endometrium. The entire mass was richly vascularized.

The uterus of the rat is apparently a rather common site for neoplastic growths. Crain (*4*) reported 12 tumors of the uterus, 2 leiomyomas, 7 mesodermal tumors, and 3 adenocarcinomas. Bullock and Curtis (*6*) reported 48 tumors of the uterus: 4 leiomyomas, 2 myosarcomas, 2 fibro-

sarcomas, 2 polymorphous-cell sarcomas, 1 spindle-cell sarcoma, 16 mixed-cell sarcomas, 1 papilloma, 10 squamous-cell epitheliomas, 4 adenocarcinomas, 1 adenoacanthoma, 1 carcinoma, 4 carcinosarcomas. Ratcliffe (*5*) reported 10 uterine tumors, which included 1 myxoma and 1 myxosarcoma. Pfeiffer (*20*, *21*) suggested a correlation between adenocarcinoma and leiomyoma of the uterus with endocrine-imbalanced rats, which he produced through testicular grafting.

Cervix.—Two fibrovascular polyps of the cervix were seen. One of them protruding through the vaginal introitus was observed ante mortem. It arose from the cervix, while the other polyp apparently arose near the junction of the vagina and cervix because both columnar and stratified squamous epithelium were present on its surface. This rat died at 20 months of age. The other rat was killed at 19 months of age. Microscopically, they were both composed of a well-circumscribed, loosely arranged mass of highly vascularized tissue.

Polyps of the uterus have been described, but we could not find any reports of polyps arising from the cervix.

Tumors of Endocrine Glands

Adrenal glands.—Pheochromocytomas occurred in 7 of our 125 rats. One corticocarcinoma was found.

Five of the pheochromocytomas were in male animals. This is probably significant since we had nearly twice as many female as male rats in the experiment. Both Gilman's and Yeakel's reports (*17*, *22*) show a significantly higher occurrence in the male rat. These 7 rats ranged in age from 22 to 30 months with an average of 22 months. Only 2 of the 7 adrenal tumors in our series were detectable grossly. Gilman *et al.* (*17*) described some pheochromocytomas as almost equaling the size of the kidney.

The pheochromocytomas we observed microscopically either completely replaced the medulla and compressed the cortex or consisted of a discrete nodule within the medulla or at the corticomedullary junction that compressed the adjacent tissue. The cells resembled normal medullary cells but had less cytoplasm and the nuclei were hyperchromatic. Mitotic figures were common. Several tumors were extremely congested with dilatation of the sinusoids. Extensive areas of hemorrhage and coagulation necrosis were seen in 2 of the tumors. The neoplastic cells were arranged for the most part in small groups, in intimate association with blood vessels. Signs of malignancy, other than an abundance of mitotic figures, were not seen, nor did we observe invasion of the capsule and perirenal tissues as described by some authors. Gilman *et al.* (*17*) have described invasion of the adrenal capsule and have seen extension along the adrenal vein and even occlusion of the posterior vena cava. They found tumor emboli in the lung but no other growth within the lung parenchyma. However, they were unsuccessful in transplanting the tumor subcutaneously.

Only 1 of our rats with a pheochromocytoma had a concurrent tumor,

an adenocarcinoma of the small intestine. Gilman *et al.* (*17*) reported on 167 male and 189 female rats from the Wistar Institute, ranging from 1 month to 2½ years of age. They found, in rats over 13 months of age, an incidence of pheochromocytoma of 50 to 76 percent in the females and 82 to 85.5 percent in the males. Two were observed in animals under 13 months of age. They associated pheochromocytomas with a high incidence of tumors of other endocrine glands: thyroid, testes, and islets of Langerhans. They also suggested a relationship of pheochromocytomas to a high incidence of chronic myocarditis and hepatic necrosis. We observed no such concomitant lesion in our animals with pheochromocytomas. There was no clinical or microscopic evidence that these tumors were secreting excessive quantities of epinephrine. Crain (*4*) described 10 medullary tumors in 786 rats. Davis *et al.* (*2*), in their report on spontaneous tumors in the Sprague-Dawley rat, did not find any neoplasms of the adrenal. Yeakel (*22*) examined the adrenals of 16 female and 15 male albino rats and 3 male and 3 female gray Norway rats, all over 700 days of age. He observed medullary hyperplasia of the adrenals in 72 percent of the males and 16 percent of the females. Saxton *et al.* (*7*) reported 5 adrenal tumors in 498 rats. Bullock and Curtis (*6*) reported 4 adrenal adenomas in 31,868 rats.

There was 1 adrenocortical carcinoma that occurred in a male animal killed at 23 months of age. This tumor mass measured 2 cm in its greatest diameter. Histologically, there was no differentiation in the normal structure of the adrenal cortex, and the various zona could not be discerned. There was no medullary tissue in the sections studied. The mass consisted essentially of epithelial cells with vesiculated, large, round, uniform nuclei and acidophilic cytoplasm with fuzzy, indistinct, cellular borders. These cells were arranged in clumps held together by fine fibrous trabeculae (fig. 3). At one point the tumor extended through the capsule and invaded the pancreas. Mitoses were not common. The center of the mass contained sheets of collagen and connective tissue with scant cellular components. The growth was well vascularized with distention of many vessels. There was no clinical or microscopic evidence that this tumor was secreting excessive amounts of adrenocortical steroids. Snell and Stewart (*23*) have reported a remarkably high incidence of adrenocortical carcinomas in the NIH-Osborne-Mendel rats. They had concomitant lesions, which indicated that some of their tumors were hormonally active; however, these investigators could not determine whether all tumors of the adrenal cortex were functional. Iglesias and Mardones (*24*) reported a carcinoma of the adrenal cortex in a female rat of the A × C strain. There are several reports of adrenocortical adenomas in the literature (*25–27*).

Hypophysis.—We observed 5 adenomas of the pituitary in rats 19 to 30 months of age (average 24.8 months). All but 1 were noted grossly at necropsy. They varied from a small 1 mm nodule to a mass 6.0 × 4.0 × 6.0 mm. Histologically, there was a variation in cell size and degree of vascularity. The cells lacking specific granules were generally of the

chromotrope type. However, single and occasionally small aggregates of basophils and acidophils were seen scattered throughout the tumor masses. The cells, for the most part, had indistinct boundaries and large, deeply staining central nuclei, very often with a prominent nucleolus. Mitotic figures were common in all the tumors. In the more vascular tumors, there were hemorrhages, and all contained a substantial number of macrophages laden with hemosiderin. In 1 tumor, multinucleated giant cells were observed that were similar to those reported by Selye (*28*) in estrogen-produced, anterior-lobe adenomas of rats. There was no clinical disturbance in these rats that could be related to the adenomas. One rat had an unexplained posterior paresis. Two rats each had a fibroadenoma of the mammary gland.

Saxton and co-workers (*7*), in reports on 234 spontaneous neoplasms in rats, found 92 pituitary tumors, all classified as chromophobe adenomas. Crain (*4*) described 11 of 200 tumors as adenomas of the pituitary. Kim, Clifton, and Furth (*11*) recorded 20 of 53 tumors as pituitary adenomas in the Wistar strain of rat. Ratcliffe (*5*) and Davis *et al.* (*2*) did not describe pituitary tumors.

Thyroid.—At necropsy, enlargement of the thyroid gland was noted in 5 animals. In all instances the tumor masses were composed of epithelial cells with leptochromatic nuclei surrounded by a pale, slightly eosinophilic cytoplasm. Mitotic figures were not common, and the cells tended to be organized into lobules (fig. 4). Small follicles containing colloid were seen within these tumors, but the cells lining the follicles had a more distinct eosinophilic-appearing cytoplasm than the tumor cells. Since the borders of the tumors were not sharply demarcated but tended to diffuse between the adjacent normal follicular structures, it appears most probable that the follicles seen within the substance of the tumors represented normal follicles that had become encompassed as the tumor enlarged.

In 4 additional rats, distinct nodules composed of the same cell type were observed within the substance of the thyroid upon microscopic examination. These nodules did not cause significant enlargement of the glands. Since only 2 to 4 random sections were made through the thyroid area of each animal, it is quite probable that small lesions of this type occurred in other animals but did not appear in the sections studied. Of the 9 animals in which this lesion was observed, it was bilateral in 4. The 9 rats ranged in age from 16 to 30 months with an average of 23 months.

At the time these neoplasms were first observed, some were interpreted as of doubtful parathyroid origin (*1*). However, it is now our opinion that all these tumors are morphologically identical to the light-cell tumor of the thyroid described by Axelrad and Leblond [4] and designated by them as type γ nodules (*29*). In older rats, large cells with leptochromatic nuclei and pale cytoplasm are seen in fairly large numbers between

[4] We are grateful to Dr. Leblond for reviewing slides of these tumors, and he agrees with this interpretation.

the follicles. Apparently it is from such so-called "light cells" that these tumors originate (*2, 9, 30–33*). In many of the older rats in our series, there appeared to be an increase in the number of these cells throughout the thyroid gland. This type of tumor occurs with increased frequency in rats on a low iodine intake. There was no reason to believe that the rats in our experiment had an insufficient iodine intake, since iodine was added in the salt mix used to compound their diets at a level of 7.5 mg KI per 1,000 g diet.

Tumors of the Kidney

We found 1 renal tumor, a nephroblastoma, in a female rat that was killed at 8 months of age. It measured 6.0 × 5.0 × 3.5 cm and had essentially replaced the left kidney. Microscopically, it was composed of a large multilobulated mass that occupied nearly all the renal cortex and medulla (fig. 5). There was a prominent, well-vascularized fibroblastic stroma. The epithelial component consisted of basophilic cells with large vesicular nuclei and single nucleoli. The glandlike epithelium mimicked renal tubules (fig. 6). There were papillary projections into some of the newly formed gland structures, which gave the impression of attempts at glomerular formation. Mitotic figures were occasionally observed. This neoplasm was similar to the rather common embryonal nephroma in swine.

Bullock and Curtis (*6*) reported 8 renal tumors: 6 embryonal carcinomas, 1 sarcoma, and 1 carcinoma. Crain (*4*) described 3 renal tumors: 1 papillary cystadenoma, 1 lipomatous hamartoma, and 1 embryonal carcinoma. Ratcliffe (*5*) reported 4 embryonal nephromas and 1 fibroadenoma. In the wild Norwegian rat, McCoy (*15*) described 11 renal tumors: 1 papilloma, 4 adenomas, 3 carcinomas, and 3 adenocarcinomas. Saxton (*7*), Eker (*34*), and Bullock and Rhodenburg (*35*) observed cases of multiple renal adenomas. Olcott (*36*) successfully transplanted a nephroblastoma to the subcutaneous tissues. Rosen *et al.* (*37*) reported an extremely high incidence of renal corticoadenomas, corticocarcinomas, and pelvic transitional-cell carcinomas in Sprague-Dawley rats given total-body X radiation. They observed a 40 percent incidence of such neoplasms in rats receiving 430 r, 29 percent in rats receiving 680 r, and none in their control rats.

Tumors of the Digestive System

Salivary gland.—We observed 1 sarcoma involving the submaxillary salivary gland in a female rat killed at 20 months of age. The salivary gland was enlarged 6 to 8 times normal size. Much of the acinar portion of the gland had been replaced by solid sheets of neoplastic cells. These cells gave the appearance of "flowing" along the course of the original intralobular spaces (fig. 7). The cells were spindle-shaped in some areas but in certain aspects resembled epithelial cells in other areas (fig.8). An occasional giant cell was seen. Mitotic figures were numerous. The

tumor invaded the adjacent musculature and adipose tissue of the neck and infiltrated directly into at least 3 lymph nodes. Special connective-tissue stains revealed an increase of reticulum and collagen fibers about the individual cells, which leaves little doubt in our minds that this was a frank sarcoma.

Comparatively few salivary-gland tumors have been reported in the rat. Curtis *et al.* (*38*) reported a parotid adenoma. Davis *et al.* (*2*) reported a carcinoma of the maxillary gland, and Ratcliffe (*5*) described a carcinoma of the salivary gland. Both McCoy (*15*) and Wooley (*39*) reported sarcomas in the submaxillary region; however, from their reports it is not clear whether the tumors involved or originated in the salivary gland.

Stomach and intestines.—We observed 1 squamous-cell carcinoma of the cardiac zone of the stomach in a 19-month-old rat that died and 1 adenocarcinoma of the small intestine in a 22-month-old rat that died.

The squamous-cell carcinoma was observed as a 5.5 × 7.0 × 3.5 cm mass extending from the wall of the stomach. Microscopically, the muscular layers were diffusely infiltrated with atypical squamous epithelial cells. Epithelial pearls were common. Portions of the neoplastic mass showed extensive keratinization in excess of that normally seen in the cardiac zone of the rat. There was a leukocytic infiltration of the stroma of this neoplastic mass with lymphocytes predominating.

The adenocarcinoma arose from the ileum and measured 2.5 × 2.0 × 2.0 cm. The lumen of the ileum was narrowed but not completely obstructed. Grossly, the tumor was solid with a necrotic center. Microscopically (figs. 9 and 10), the viable portion of the tumor was dispersed throughout the necrotic tissue and consisted of acini arranged in bizarre patterns. Numerous mitotic figures were present in the epithelial cells. Many of the necrotic areas were infiltrated by polymorphonuclear leukocytes. These groups of acinar cells infiltrated the lamina propria and muscularis but did not extend through the serosal surface of the ileum. Many of the necrotic areas and the muscularis of the ileum were infiltrated by numerous polymorphonuclear and mononuclear leukocytes.

Tumors of the gastrointestinal tract, especially malignant ones, are rare in rats. Only 6 adenocarcinomas have been reported in the literature: 2 involved the stomach, 1 the cecum, and 3 the colon (*4*, *6*, *40*). No malignant epithelial neoplasm of the small intestine has previously been reported. Saxton (*7*) has reported a squamous-cell carcinoma of the stomach, and Crain (*4*) a squamous-cell carcinoma of the esophagus. Sarcomas, fibromas, and adenomas of the gastrointestinal tract have been described.

Pancreas.—We observed 1 pancreatic neoplasm, an islet-cell adenoma in a 22-month-old rat that was killed. Histologically, this appeared similar to normal islet tissue but was manyfold larger, measuring 4 mm in its largest diameter. Occasional mitotic figures were seen. Since it was an incidental finding on microscopic examination, we have no way of knowing whether this was a functional adenoma.

Tumors of the Brain and Associated Structures

We observed 4 neoplasms of the brain and its related structures. To our knowledge the only previously recorded brain tumors are the glioblastoma reported by Saxton (*7*), the meningioma reported by Gilbert, Gillman, Loustalot, and Lutz (*19*), and the glioma reported by Highman and Altland (*41*). Those we observed were an ependymoma, a papilloma of the choroid plexus, a meningioma, and a pinealoma.

The ependymoma was found in an 11-month-old rat that died. Grossly, it appeared as a nonencapsulated mass in the area of the third ventricle, on the right side, anterior to the optic chiasm. Microscopically, the tumor was highly vascularized, with areas of hemorrhage and central cystic degeneration. There were numerous gitter cells surrounding the hemorrhagic areas, many containing phagocytized erythrocytes and others heavily ladened with hemosiderin. Many tumor cells in the center of the mass had distinct cellular boundaries and appeared similar to ependymal cells. Near the periphery of the lesion, adjacent to the lateral ventricle, the tumor cells were less differentiated, with indefinite borders and large nuclei (figs. 11 and 12). Several pseudorosettes were noted throughout the tumor. Mitotic figures were numerous.

The papilloma of the choroid plexus, occurring in a 22-month-old rat that died, was not recognized at the time of necropsy. Histologically, there was a focus of neoplastic tissue within the wall of the third ventricle that projected into the ventricular lumen (fig. 13). The mass was composed of cells with large hyperchromatic nuclei and eosinophilic cytoplasm (fig. 14). The tissue was well vascularized. There was no apparent rosette formation.

The meningioma was found in a 17-month-old rat that died. Basically, it was made up of interlacing strands of fibroblastic tissues, which in some areas were arranged in whorls. Some areas were highly cellular, but there was little evidence of mitotic activity. Although there was no invasion of the brain tissue, the tumor grossly extended through the cribriform plate and microscopically infiltrated the olfactory tracts. Psammoma bodies were seen throughout the neoplasm (fig. 15).

In the rat, neoplasms of the pineal body are apparently very rare. At the time of necropsy we observed such a neoplasm between the two cerebral hemispheres at their posterior portion just anterior to the sagittal sinus in a 16-month-old rat that was killed (fig. 16). Histologically, it was made up of large, pale-staining parenchymal cells and smaller round, dark-staining cells (fig. 17). Mitoses were very numerous and the mass encroached on the surrounding brain tissue (figs. 17 and 18). Brain sand (acervulus cerebri or corpora arenacea) was present within the tumor.

Tumors of Skin and Subcutaneous Tissues

We observed only 2 tumors of the skin and nonglandular subcutaneous structures. One was a papilloma of the skin of the cervical area measuring $3.7 \times 2.6 \times 2.0$ cm in an 18-month-old rat that was killed. In spite of

its large size there was no evidence of infiltration, and it was not essentially different from those already reported in the literature. The other was a fibrosarcoma that arose in the subcutaneous tissues of the right cervical region in a 9-month-old rat that was killed. This tumor measured 3.0 × 3.7 × 2.5 cm. The neoplasm consisted of a roughly circumscribed, multilobulated tumor mass composed of collagen and connective-tissue cells. The nuclei of the fibroblasts showed considerable pleomorphism, and mitoses were numerous. The tumor invaded the adjacent skeletal musculature. Variable degrees of cellular density were observed within the tumor, being less cellular and more collagenous at the center of the lobules and of greater cellularity at the periphery and in those portions invading the underlying musculature.

Tumors of the skin and adnexa and nonglandular, subcutaneous structures have accounted for a large percentage of neoplasms reported by some investigators. Bullock and Curtis (*6*) reported 121; Ratcliffe (*5*), 22; Crain (*4*), 4 of 200 tumors; and Davis *et al.* (*2*), 1.

Tumors of Lymphoid Tissues

The single incidence of malignant lymphoma that we observed was in a 20-month-old rat that was killed, and involved the mediastinal tissues, uterus, periovarian tissues, ovary, perirenal tissues, periadrenal tissues, pancreas, small intestine, stomach, and visceral peritoneum. Mitotic figures were very numerous. This tumor did not differ essentially from similar tumors observed in domestic animals (fig. 18).

Some investigators have reported a rather high incidence of malignant lymphoma: 91 by Saxton (*7*), 78 by Bullock and Curtis (*6*), and 41 by Crain (*4*). All authors, however, have not noted such a high incidence. Ratcliffe (*5*) reported only 1, and none occurred in the series reported by Davis *et al.* (*2*) and McCoy (*15*).

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PLATES

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PLATE 157

FIGURE 1.—Granulosa-cell tumor. This tumor was primarily of a solid medullary type; however, there were occasional open, fluid-filled spaces. Hematoxylin and eosin. × 475

FIGURE 2.—Hemangioendothelioma, oviduct. The lamina propria was replaced by large, blood-filled spaces. Hematoxylin and eosin. 6× microtessar.

FIGURE 3.—Adrenocortical carcinoma. Epithelial cells were arranged into small clumps separated by thin strands of connective tissue. Hematoxylin and eosin. × 516

FIGURE 4.—Light-cell adenoma, thyroid. Tumor cells were generally arranged in lobules. Normal thyroid to *left*. Hematoxylin and eosin. × 500

FIGURE 5.—Nephroblastoma, kidney. This tumor was composed of large quantities of connective tissues and an epithelial component growing in solid sheets, open tubules, and glomerular structures. Hematoxylin and eosin. 6× microtessar.

FIGURE 6.—Nephroblastoma, kidney. Higher power of figure 5. Epithelial cells are seen in a solid group (*lower left*) and forming tubules (*upper*). Hematoxylin and eosin. × 516

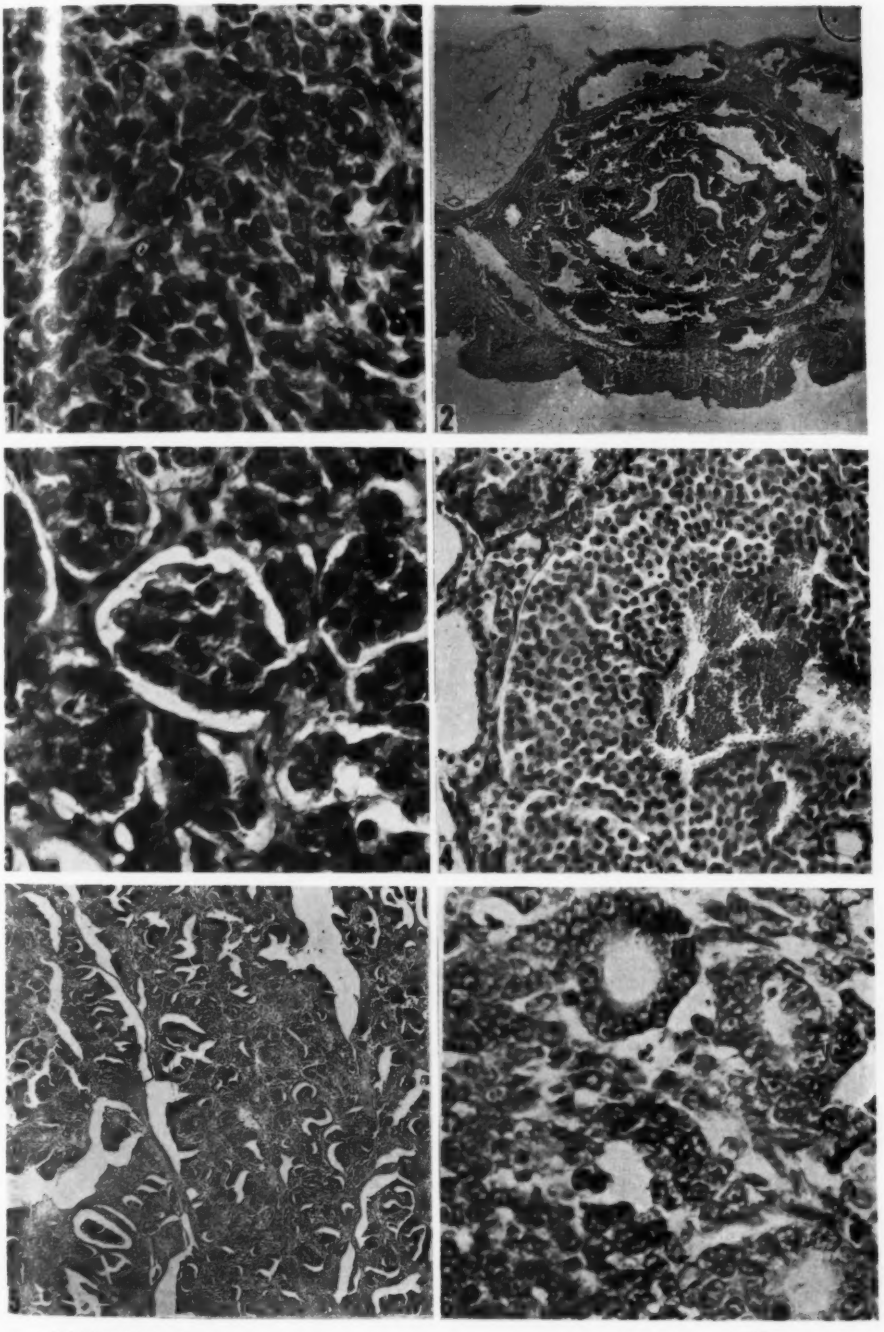

PLATE 158

FIGURE 7.—Fibrosarcoma, salivary gland. Sarcoma that grew within gland divided it into tumor-infiltrated lobules. Three infiltrated lobules are shown. Hematoxylin and eosin. 6× microtessar.

FIGURE 8.—Fibrosarcoma, salivary gland. Higher power of figure 7. Some remaining glandular structures are present in *upper-right* quadrant. Hematoxylin and eosin. × 200

FIGURE 9.—Adenocarcinoma, small intestine. This tumor measured 2.5 × 2.0 × 2.0 cm and nearly occluded the lumen of the ileum. Glandular structures are seen infiltrating the muscularis (*upper right*). Most of this tumor was necrotic. Hematoxylin and eosin. 48 mm microtessar.

FIGURE 10.—Adenocarcinoma, small intestine. Higher power of figure 9. Groups of differentiated epithelial cells that have formed an acinar pattern. Hematoxylin and eosin. × 225

FIGURE 11.—Ependymoma, brain. Tumor is encroaching on normal brain substance. Hematoxylin and eosin. × 85

FIGURE 12.—Ependymoma, brain. Higher power of figure 11, with a mitotic figure in *center*. Hematoxylin and eosin. × 500

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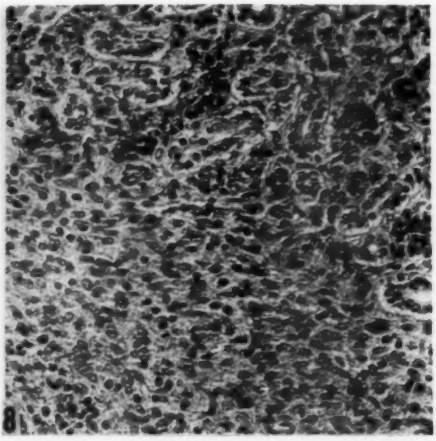
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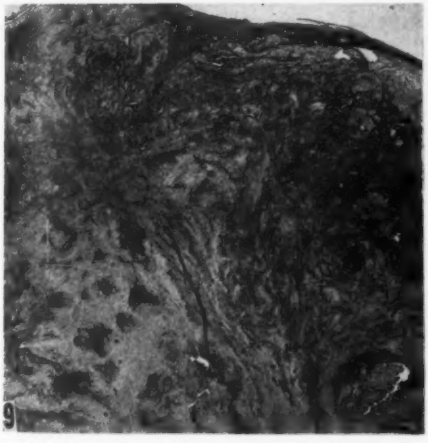
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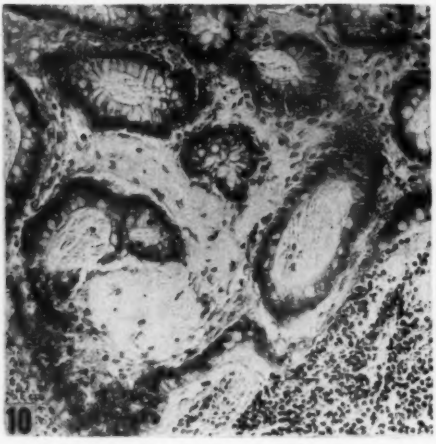
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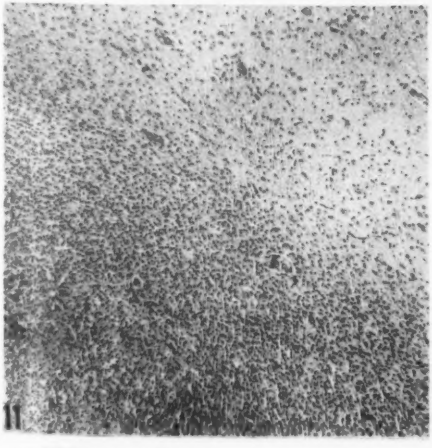
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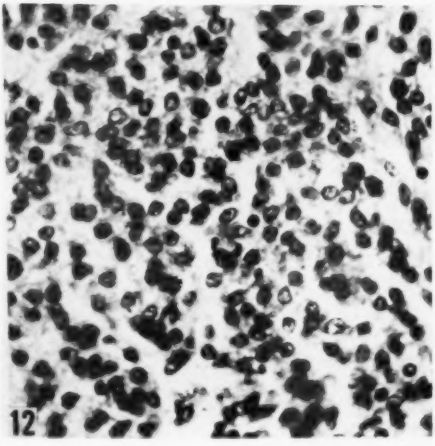
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PLATE 159

FIGURE 13.—Papilloma, choroid plexus. Tumor is seen within lateral ventricle. Hematoxylin and eosin. 6× microtessar.

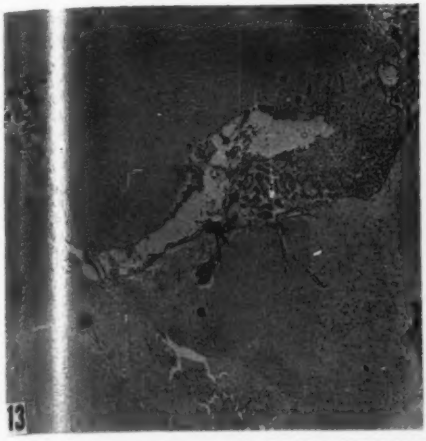
FIGURE 14.—Papilloma, choroid plexus. Higher power of figure 13. Growth contained both papillary and solid areas. Hematoxylin and eosin. × 350

FIGURE 15.—Meningioma, brain. Psammoma body is seen. Other areas of tumor contained more definite whorled pattern. Hematoxylin and eosin. × 400

FIGURE 16.—Pinealoma, brain. Tumor is seen between two cerebral hemispheres compressing adjacent brain tissues. Hematoxylin and eosin. 48 mm microtessar.

FIGURE 17.—Pinealoma, brain. Higher power of figure 16 that shows junction of tumor and brain tissue. Hematoxylin and eosin. × 225

FIGURE 18.—Pinealoma, brain. These large parenchymal cells predominate throughout tumor. Mitotic figures are numerous. Hematoxylin and eosin. × 516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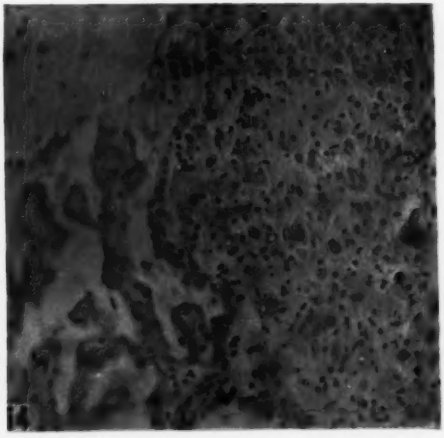
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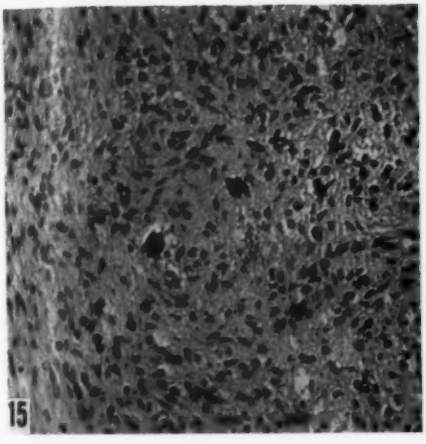
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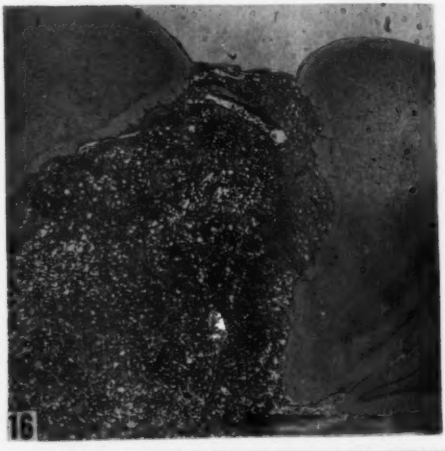
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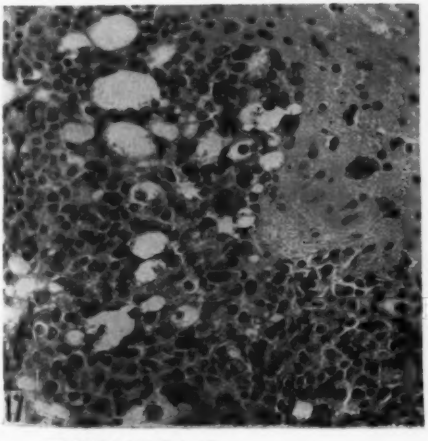
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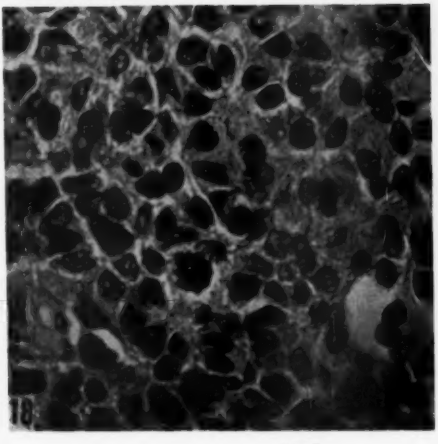
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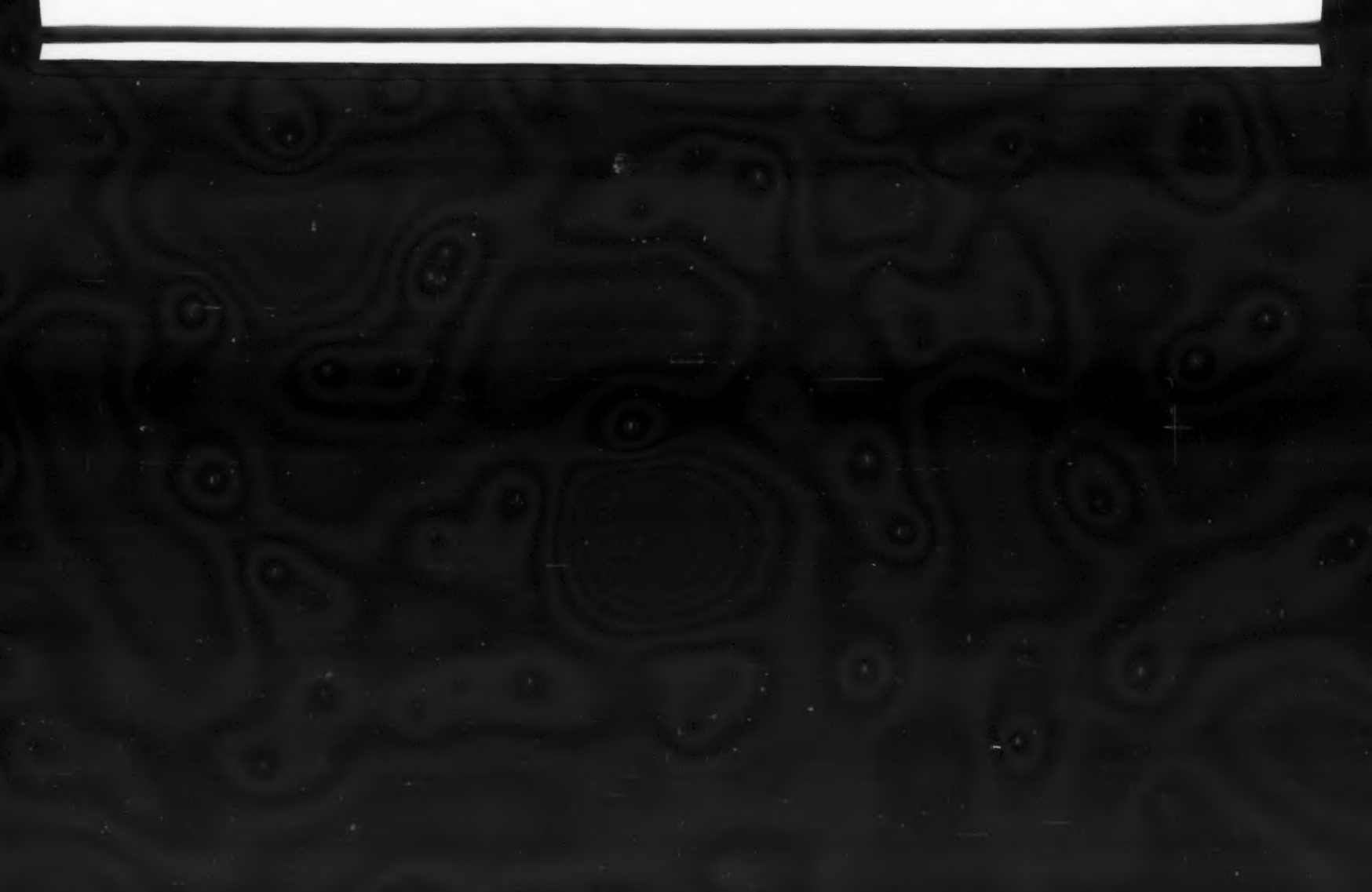

Skin Homograft Tolerance in Long-Lived Radiation Chimeras and Its Abolition by Injected Lymphoid Cells [1, 2]

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SUMMARY

(C57L × A) F_1 hybrid mice (LAF_1) were exposed to a lethal dose of X rays (870 r) and then received intravenous injections of mouse bone marrow cells obtained from genetically foreign normal donors (C3H strain). The mice that had survived for 1 year or more after this treatment were then challenged with tail-skin grafts to evaluate their immunological state. LAF_1 and C3H-type skin grafts (corresponding to the host and the marrow donor strains, respectively) were accepted by almost all the recipients. BALB/c- or DBA/2-type skin grafts (foreign to both the host and marrow donor strains) were rejected. These long-lived mice, known to be radiation chimeras, are therefore capable of a homograft response, but are tolerant to skin grafts of host (LAF_1) or of marrow donor (C3H) genotype. The tolerance to C3H skin grafts was abolished, and the C3H skin grafts were rejected 2 to 3 months after inoculation with lymphoid cells obtained from either normal or sensitized (anti-C3H) LAF_1 donors. Other chimeras, receiving injections of irradiated LAF_1 lymphoid tissue, maintained tolerance to the C3H skin grafts. To test for the presence of immunologically active C3H-type cells in these chimeras, cells from their lymphoid tissues were injected into sublethally irradiated (C3H × DBA/2)F_1 hybrids, and the signs of wasting and death known to follow the injection of parental strain C3H cells (but not LAF_1 cells) were observed. With this system, C3H lymphoid cells were present in the tolerant mice. In contrast, these cells were not present in the mice that were no longer tolerant to C3H grafts.—J. Nat. Cancer Inst. 27: 1059–1067, 1961.

HOMOLOGOUS [3] RADIATION chimeras (irradiated mice bearing a graft of genetically foreign bone marrow cells) have an altered immunological response in that they accept homologous transplants taken from mice corresponding to the bone marrow donor strain. This apparent tolerance has been demonstrated with tissue transplants, such as skin (*1–3*), strain-specific tumors (*4*), and lung (*5*). On the other hand, these

[1] Received for publication May 16, 1961.

[2] This study was supported through funds provided by the Bureau of Medicine and Surgery, U.S. Navy Department, and the Office of Civil and Defense Mobilization. The opinions and assertions contained herein are those of the authors and are not to be construed as official or as reflecting the views of the Navy Department.

[3] Gorer (Nature, London 189: 1024, 1961) has criticized the use of the term "homologous" and has proposed in its place the word "allelogenic"; in place of "isologous" he suggests the term "isogenic."

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chimeras are able to reject heterologous rat lung grafts (*5*) and develop a titer in response to sheep or human erythrocytes (*6*). Recently, it was shown in this laboratory that long-lived radiation chimeras contained immunologically competent cells of donor origin in their lymphoid tissues, and these cell populations were specifically tolerant of host-type cells (*7*).

Our study extends this observation of specific tolerance by means of tail-skin homografts on the intact chimera. In addition, it correlates the induced abrogation of this tolerance with disappearance of the lymphoid cells of donor origin from these chimeras.

METHODS

Long-lived radiation chimeras.—Three-month-old (C57L × A/Hc)F_1 hybrids (so-called LAF_1 mice, bred at this laboratory), were exposed to 870 to 880 r (100 r greater than LD99) of X radiation and protected with an intravenous injection of homologous bone marrow ($5–7 \times 10^6$ nucleated cells suspended in Tyrode's solution) from young adult C3H donors. Isologous "chimeras" received 5 to 7×10^6 bone marrow cells from LAF_1 donors. The animals were maintained on Purina fox chow and water *ad libitum* in cages holding up to 10 animals. About 70 percent of the mice that had received C3H bone marrow died 1 to 4 months later from secondary disease. The long-term survivors, when grafted, had a healthy appearance and a good growth of unpigmented hair. The females weighed 22 to 26 g, and the males 25 to 30 g.

Tail skin grafts.—The method used paralleled that described by Bailey and Usama (*8*). The tail-skin donors were LAF_1, from our animal colony, C3H/Crgl,[4] BALB/c Jax,[5] and DBA/2 Jax.[5] The graft bed on an ether-anesthetized chimera was prepared by the removal, with a razor blade, of a thin slice of tail skin, 1 to 2 mm wide and 3 to 4 mm long. Skin from the donor's tail (anesthetized with Nembutal) was then cut to fit the graft bed. The skin graft was turned around so that the hair lay opposite that of the recipient, and then pressed firmly onto the graft bed. Skin from the LAF_1 donor was always grafted first, nearest the base of the tail; the C3H skin graft was placed in the middle, and the foreign BALB/c or DBA/2 skin was grafted last, nearest the tip of the tail. The glass tubes, used to protect the grafts, were left on for 24 hours. If they were left in place longer, infection and depilation of the grafts and the host's tail were likely to occur.

The grafts were observed 2 to 3 times weekly with a dissecting microscope at 10× magnification. C3H skin grafts could be easily distinguished by the dark pigmentation of the skin and hair. The hair color of BALB/c (white) and DBA/2 (gray) was also distinguishable under the microscope.

[4] Purchased from the Cancer Research Genetics Laboratory, University of California, Berkeley, Calif.
[5] Purchased from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Me.

The following sequence of events characterized rejection of a tail-skin homograft not tolerated by healthy chimeras:

Day 2.—A yellow, crusty scab surrounded the periphery of the graft. The crust sometimes overlaid the entire graft, but it was sloughed off at least by the 10th day, which left the donor skin and hair in place.

Day 4.—The homografts appeared to be "taking," though there sometimes was evidence of inflammation.

Day 6.—Inflammation usually was noted.

Day 10.—Inflammation occurred together with hemorrhagic breakdown and some scabbing.

Day 14.—The graft usually still had hair but was a dry scab that could readily be picked off. ***Grafts in this condition were regarded as rejected.***

Day 18.—The graft was usually gone, as evidenced by disappearance of the graft hair. The graft bed was sometimes inflamed or scabbed over.

Day 25.—The graft bed was bare and healed. Hair usually did not grow again in scar area unless the hair follicles of the host were not removed completely during grafting. In this event, the host hair was distinguished from graft hair by the orientation of its growth.

The evidence for rejection of established C3H skin grafts after the abolition of tolerance (to be described later) was less obvious. The disappearance of C3H hair and skin pigments over a period of 3 or more weeks and, perhaps, slight inflammation were the only signs of rejection under these conditions.

Skin grafts that were accepted did not elicit any inflammatory response by the 6th day and showed no signs of breakdown or scabbing. At times, a flap of a graft did not adhere properly and dried to a scab, but it was later sloughed, which left the nourished portion of the graft in place. C3H grafts usually maintained a good stand of hair, but the LAF_1 grafts frequently were bald by the 2d week. The fate of these grafts could not be confirmed until 1 to 2 months later when new LAF_1 hair, growing in a direction opposite to that of the host, appeared in the accepted grafts.

In general, a C3H skin graft was considered accepted if it maintained or grew hair for 20 days or more after grafting and if there was no inflammation. If no hair was present, but skin pigments were noted, the graft was considered as a questionable "take."

Determination of C3H transplantation immunity isoantigen.—The method has been described previously (*5*, *7*). It depends on the inability of C3H bone marrow to protect lethally irradiated LAF_1 recipients that had been sensitized with C3H isoantigen (nucleated cells) before irradiation. The antigen in the long-lived chimeras is presumed to be from C3H cells (*5*).

Demonstration of C3H-type lymphoid cells in the chimeras.—It has been shown that the administration of parental lymphoid cells into sublethally irradiated hybrid mice will result in their death (*9*, *10*). Lymphoid cells from donors foreign to the hybrid are not lethal. To show the presence of C3H reactive cells in the lymphoid tissues of a chimera, the spleen and lymph nodes were removed, lightly homogenized in cold Tyrode's solution, and aliquots with 30 to 50 million nucleated cells were injected intraperitoneally into (C3H × DBA/2)F_1 hybrid mice[6] ($C3D2F_1$) that had been

[6] Purchased from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Me.

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exposed to 500 r of X rays. Other sublethally irradiated $C3D2F_1$ mice, which had been similarly exposed, received lymphoid cells from normal LAF_1 or C3H (parental) donors. The hybrids were then observed for signs of wasting and death.

RESULTS

Homograft Tolerance in Long-Lived Chimeras

Four groups of long-lived homologous bone marrow chimeras and one group of old isologous bone marrow "chimeras" were grafted with tail skin from normal LAF_1, C3H, and either BALB/c or DBA/2 donors. The fate of these grafts is shown in table 1. In the homologous bone marrow chimeras, the C3H grafts were accepted by all the recipients, with the possible exception of one. In that one instance, hair did not grow from the graft, but the site was pigmented. The second group of homologous chimeras, which was observed for only 16 days, was probably killed too early to be certain of the acceptance of the C3H grafts, though no apparent reaction to the grafts was evident.

TABLE 1.—Fate of isologous and homologous tail-skin grafts on long-lived radiation chimeras

Homologous chimeras* (age in days post bone marrow)	Presence of C3H transplantation isoantigen (No./total)	Skin grafts (No. "takes"/No. grafted)		
		C3H	LAF_1	BALB/c or DBA/2
390	4/4	3/4 (43)†	4/4 (43)	0/4
470	4/4	4/4 (16)	3/4 (16,	0/4
355	4/4‡	4/4 (56)	4/4 (56)	0/4
446	Not tested	6/6 (57)	6/6 (57)	0/6
Isologous "chimeras"				
411	0/2	0/4	3/4 (31)	0/4

*Chimeras were LAF_1 hybrids exposed to 880 r of X rays and protected with bone marrow from C3H (homologous) or LAF_1 (isologous) donors.

†Numbers in parentheses indicate the number of days skin graft "takes" were observed before mice were killed. Rejected grafts were sloughed off in 13 to 14 days.

‡Immunologically competent cells of donor (C3H) origin were detected in this group of chimeras (7).

In the isologous marrow "chimeras," the C3H skin homografts were inflamed by the 8th day and were scabbed or gone by the 13th day.

In the homologous and isologous chimeras, LAF_1 skin grafts were accepted in all but 2 recipients. Of these 2, one died and the other was killed before the fate of the grafts could be evaluated. In all groups, both the homologous (C3H) and the isologous (LAF_1) marrow recipients rejected BALB/c or DBA/2 grafts within 14 days.

The acceptance by the homologous marrow chimeras of host- and donor-type skin grafts indicates that a state of tolerance existed in them. That this tolerance was specific and not due to immunological unresponsiveness was shown by the ability of these animals to reject homografts foreign to both the marrow donor and host genotype.

A C3H isoantigen which elicits transplantation immunity was found in the lymphoid tissues (spleen and lymph nodes) of all the chimeras tested. No C3H isoantigen was found in the lymphoid tissue of the isologous marrow recipients tested.

Abrogation of Tolerance With Normal LAF_1 Spleen Cells

Eight long-lived homologous chimeras were grafted with LAF_1, C3H, and BALB/c skin. All BALB/c grafts were rejected by 20 days as shown in table 2. Seven of the 8 C3H grafts "took." The 8th graft was a questionable "take," bald but pigmented. Six of the 8 LAF_1 grafts "took," while the remaining 2 also were questionable "takes." Twenty-four days after grafting, 5 of the 8 tolerant chimeras received intraperitoneal injections of normal LAF_1 spleen cells (equivalent to ½ spleen each) prepared by homogenization of the spleen in Tyrode's solution. The 3 remaining animals received injections of another aliquot of the homogenate that had been exposed *in vitro* to 1000 r of X rays. The appearance of the skin grafts was checked 2 to 3 times weekly thereafter.

Two of the recipients that received nonirradiated LAF_1 spleen cell injections died 24 days later, with the C3H and LAF_1 grafts apparently still intact. Of those remaining, one lost its C3H graft 24 days after the nonirradiated spleen injection, another lost the hair and pigment from its C3H graft about 2 months after injection after a period of slight inflammation. The C3H graft on the third recipient was bald and depigmented 2½ to 3 months after the spleen injection, but there was no sign of an inflammatory response. In general, the C3H grafts were apparently slowly sloughed, as evidenced by the disappearance of the hair and of the dark pigments of C3H skin over a period of 3 weeks or more.

In those animals that received irradiated LAF_1 spleen cells, the status of the C3H grafts remained the same as before injection; *i.e.*, 2 had positive C3H skin "takes" while 1 was a questionable "take." One of the 3 LAF_1 grafts in this group still was maintaining good hair growth at 166 days post grafting, but the other 2 LAF_1 grafts had slowly become bald and were apparently gone.

Abrogation of Tolerance With Sensitized LAF_1 Spleen Cells

Eleven long-lived homologous chimeras were grafted with LAF_1, C3H, and BALB/c tail skin. The BALB/c grafts on all recipients were gone or were dried scabs by 14 days after grafting (table 2). All the C3H and LAF_1 grafts "took" and were covered with hair 65 days after grafting. At that time, 6 tolerant animals received intraperitoneal injections of spleen cells (equivalent to ½ spleen each) from sensitized (anti-C3H) LAF_1 donors prepared as a homogenate in Tyrode's solution. We sensitized these spleen cell donors by giving each of them intraperitoneal injections of cells from ¼ of a C3H spleen at 1-week intervals for 4 weeks. The remaining grafted chimeras received a similar amount from another aliquot of sensitized spleen cells that had been exposed *in vitro* to 1000 r of X rays.

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TABLE 2.—Abrogation of tolerance to C3H skin grafts by injection of LAF_1 spleen cells into long-lived radiation chimeras

LAF_1 spleen cell inoculum*	Fate of tail-skin grafts				
	Before injection			After injection	
	C3H (No. "takes"/No. grafted)	LAF_1 (No. "takes"/No. grafted)	BALB/c (No. "takes"/No. grafted)	C3H (No. "takes"/No. grafted)	LAF_1 (No. "takes"/No. grafted)
Normal (given 24 days post grafting)	5/5	4/5	0/5	0/3† (24–98 days)‡	2/3 (33 days)‡
Irradiated normal	2/3§	2/3§	0/3	2/3§	1/3 (31–34 days)‡
Sensitized (anti-C3H) (given 65 days post grafting)	6/6	6/6	0/6	1/6 (55–74 days)‡	6/6
Irradiated sensitized	4/5	3/5	0/5	4/5	3/5

* Recipients were LAF_1 hybrids exposed to 870 r of X rays and protected with C3H bone marrow. They had survived 355 days post bone marrow when grafted.

† Two chimeras died 24 days post spleen injection.

‡ Numbers in parentheses indicate the range survival time of the grafts after spleen injection. Grafts that were not rejected were observed for a period of 166 days post grafting.

§ Remaining graft was a questionable "take."

In the 6 animals that received cells from sensitized donors, 3 showed slightly inflamed C3H grafts by the 10th day after cell injection. In one, reddening continued until the graft was gone, but in the other 2, the inflammation faded even though the hair was eventually shed. The 3 graft sites were bare, with no apparent pigmentation, 2 months after administration of the sensitized cells. In 2 of the 3 remaining animals that received sensitized spleen cells, the hair and pigment had disappeared without inflammation or scabbing 2 months after inoculation. This sequence of events resulting in C3H graft rejection was similar to that observed in the chimeras that had received normal, nonsensitized cells, except for the early inflammatory response seen when the sensitized cells were rejected.

The grafts on the chimeras given irradiated, sensitized spleen cells remained unchanged; the 4 that had C3H skin-graft "takes" still retained these grafts in good condition after 166 days. The 3 LAF_1 skin-graft "takes" also remained intact during this period.

Demonstration of C3H Lymphoid Cells in the Chimeras

The chimeras that received injections of LAF_1 spleen cells (table 2) were killed 185 days after grafting (540 days post bone marrow infusion), and their lymphoid tissues were tested for the presence of C3H cells. Between 166 and 185 days post grafting, 2 normal, 2 sensitized, and 4 irradiated spleen cell recipients had died. The results for the individual surviving animals are given in table 3.

When sublethally irradiated $C3D2F_1$ hybrid mice received injections of lymphoid cells from known, normal C3H strain donors (*i.e.*, parental strain), 9 out of 10 mice succumbed. Injection of lymphoid cells from

TABLE 3.—Correlation of abrogation of skin homograft tolerance with rejection of donor-type lymphoid cells in radiation chimeras

Chimera No.	LAF_1 spleen cell inoculum injected	Fate of C3H skin grafts	Presence of C3H lymphoid cells* (No. dead/total)
1	Sensitized cells	Rejected	0/4
2	Sensitized cells	Rejected	0/4
3	Sensitized cells	Rejected	0/4
4	Normal cells	Rejected	4/4
5	Sensitized cells	Not rejected	4/4
6	Irradiated cells	Not rejected	4/4
7	Irradiated cells	Not rejected	4/4
8	Irradiated cells	Not rejected	4/4
9	Irradiated cells	Not rejected	4/4
Normal lymphoid cells of known genotype:		C3H	9/10
		LAF_1	0/10

* Test system: (C3H × DBA/2)F_1 hybrids exposed to 500 r of X rays and receiving intraperitoneal injections of 30 to 50 million lymphoid cells from the chimeras or from mice of known genotype. The hybrids are killed under these conditions by parental type C3H lymphoid cells.

normal LAF_1 donors elicited no deaths. When sublethally irradiated $C3D2F_1$ hybrid mice received injections of lymphoid cells derived from the tolerant chimeras (table 3, chimeras #5–9), all wasted and died. This indicated that immunologically competent C3H cells were present in these chimeras. In contrast, injection of lymphoid cells from 3 out of 4 of the mice, in which tolerance to C3H skin grafts had been abolished, did not kill the F_1 hybrids. In the one exception (table 3, chimera #4), the C3H skin graft had remained intact for 94 days post spleen inoculation. All other C3H grafts had been rejected before 74 days (table 2). Also, it should be mentioned that this was the only chimera tested that received injections of normal, nonsensitized, LAF_1 spleen cells. Therefore, the actual immunological status of this particular chimera is somewhat in doubt. It should be emphasized at this point that each chimera should be considered on an individual basis with respect to its immunological response. It is concluded that these tolerant radiation chimeras all possess C3H-type lymphoid cells and that these cells, as well as the C3H skin grafts, are rejected after the injection of sensitized, host-type (LAF_1) lymphoid cells into these chimeras.

DISCUSSION

The acceptance of donor-strain (C3H) and host-strain (LAF_1) skin grafts by long-lived radiation chimeras suggests that a state of tolerance must exist in them. In addition, these animals are immunologically competent, as evidenced by the rejection of skin homografts foreign to both the marrow donor and the host strain. Furthermore, we have shown here and in our previous series (*5*, *7*) that donor-type transplantation isoantigens are present in the lymphoid tissues of these late homologous radiation chimeras. By independent means, it has been demonstrated also that immunologically competent cells of donor origin (C3H) are present in

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these tolerant chimeras [*see also* (*7*)]. Therefore, specific tolerance on the part of the lymphoid cells of donor type toward tissues of the host is well founded (*7*). However, the question of the return of the host's own immune cell system and of the possible tolerance on the part of these cells with respect to the donor (that is, mutual tolerance) is still a matter to be investigated further.

Concepts regarding the development of tolerance on the part of the donor cells in the long-lived chimera (*7*) are based in part on the Burnet-Lederberg clonal selection theory of immunity (*11*). According to this theory, maintenance of tolerance requires the persistence of the isoantigen. Nossal (*12*) has indicated, indeed, that the antigen to which the recipient is tolerant must be present in order that actively acquired tolerance be maintained. Certainly, host isoantigens are present in the radiation chimera, and this may account for the persistence of specific tolerance on the part of the donor (C3H) cells toward the host. These tolerant C3H cells are evidently destroyed after injection of host-type lymphoid cells from normal or sensitized nonirradiated donors, and immunity to C3H tissues returns. This immunity probably stems from the injected host-type lymphoid cell graft, but it could theoretically also arise from the original host tissue cells which, through mutation (*11*), in the absence of donor isoantigens became immune. Therefore, from the evidence thus far available, apparently the tolerance of these chimeras with respect to C3H skin grafts is referable to a total or partial replacement of the host's immune system with C3H cells that are specifically tolerant of the host. Partial replacement implies the simultaneous presence of donor- and host-type immunogenic cells (possibly in different proportions in different chimeras) mutually tolerant to one another.

In these experiments, it required from 1 to 2 months for tolerance to the donor C3H tissue to be abolished after the injection of normal or sensitized host-type spleen cells. In their experiments with actively acquired tolerance in mice, Billingham *et al.* (*13*) gave considerably greater numbers of normal host-type lymph-node cells and noted complete breakdown of the grafts in 1 month or less. With sensitized lymph-node cells, they observed a rapid breakdown of the grafts within 2 weeks. Trentin (*14*) observed signs of breakdown in mice with actively acquired tolerance 18 days after the administration of normal host-type spleen or lymph-node cells. With the present long-lived homologous chimeras, however, this rapid rejection was not observed. Although the basis for these differences may be merely a quantitative one (*i.e.*, in terms of numbers of lymphoid cells injected), one must be aware also that the radiation chimera has probably undergone considerably more "alteration"—the host having been subjected to 880 r of X rays—than the mice given injections at birth, and also that these radiation chimeras are old and near the end of their lifespan. Therefore, their homograft response may be less intense than that of younger mice. Trentin (*14*) also observed the breakdown of tolerated Cb-grafts 18 days after the administration of host-type spleen or lymph-node cells into irradiated CBA hosts bearing (Cb $\times$ CBA)F_1 bone

marrow transplants of about 220 days' duration. His experiments differ from ours in that his host strain is parental to the hybrid bone marrow transplant, whereas ours is homologous. In the former, the donor is tolerant of the host on a genetic basis, while in the latter, donor-to-host tolerance was induced.

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Studies on the Mechanism of Isoantigenic Variant Formation in Heterozygous Mouse Tumors. I. Behavior of *H*–2 Antigens D and K: Quantitative Absorption Tests on Mouse Sarcomas [1, 2]

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SUMMARY

Previous studies on the MSWB sarcoma of (A × A.SW)F_1 origin have shown that its cells contained antigens D and K of the *H-2* system. It was possible to select isoantigenic variants in appropriate animal hosts. These hosts either lacked K but contained D, or lacked D but contained K. There were two types of variants isolated, as judged by their transplantation characteristics—they either behaved as if they had lost K and still contained D, or had lost both D and K. It was impossible to isolate a third type, which behaved like it had lost D but still had K. This paper extends these results to 3 tumors of (A × A.SW)F_1 origin and 2 of (A × A.CA)F_1 origin. A total of 33 variants was isolated. The transplantation data were in full agreement with the previous preliminary findings. Furthermore, the variants were analyzed for the actual presence of antigens D and K in their cells by a quantitative absorption technique. The serologic analysis completely agreed with the expectations based on the transplantation tests. For correct interpretation of the data, it was essential to exclude possible antigenic contribution of host cells. This was done by using newborn, irradiated hosts containing neither D nor K. One possible explanation of these findings is that isoantigenic variant formation is based on a mechanism involving mitotic crossing over, but further studies are required to establish this definitely.—J. Nat. Cancer Inst. 27: 1069–1093, 1961.

PREVIOUS STUDIES (*1–6*) have shown it possible to isolate variant sublines from tumors of F_1 hybrids derived from the crossing of 2 coisogenic resistant (IR) lines of mice (*7*) which differ at the histocompatibility-2 (*H-2*) locus. The variants were specifically compatible with one parental strain and apparently had lost the *H-2*-determined isoantigens specifically derived from the opposite parental type. Such variants were stable and irreversible, and the missing antigenic phenotype was not re-established despite prolonged passage in the genotype of origin.

Various mechanisms, both genetic and epigenetic, have been considered

[1] Received for publication May 22, 1961.

[2] This work was supported by grants from the Swedish Medical Research Council and the Swedish Cancer Society, and by grant C-3700 from the National Cancer Institute, National Institutes of Health, Public Health Service.

[3] The valuable technical assistance of Mrs. Maj-Lis Solberg, Miss Birgitta Petterson, and Mrs. Marianne Myrenberg is gratefully acknowledged.

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as explanations of this phenomenon. Genetic mechanisms have been regarded more probable for a number of reasons (*4–6*), with mitotic crossing over considered the most likely. This mechanism would lead to apparent antigenic losses and no gain of new specificities, in agreement with what was actually found. It would yield homozygous rather than hemizygous variants. In his work with lymphomas of F_1-hybrid origin, Hellström (*6*) actually found that variants selected for compatibility with one parental strain (P_1) became phenotypically similar to homozygous cells (of P_1 origin) with regard to their sensitivity to the cytotoxic action of anti-*H-2* isoantibodies (of the type P_2-anti-P_1, P_2 being the opposite parental strain that entered the cross) and were distinctly different from the less sensitive heterozygous cells of the original F_1 lymphoma from which they had been derived. Also, the P_1 variants grew more specifically and were less liable to take in the opposite parental strain (P_2) than the original F_1 tumor, similar to homozygous tumors. Analogous findings were obtained with a number of F_1 carcinomas and sarcomas and their variants (*5*). This, together with the complete failure of persistent attempts to "switch" established variants compatible with one parental strain to the opposite parental type, would again be in agreement with a homozygous rather than a hemizygous constitution (unless the loss of both *H-2* factors would be lethal). There is thus a phenotypic similarity between the variants and cells of homozygous origin. However, such phenotypic similarity cannot be taken as evidence of a homozygous genotype. A genetically hemizygous cell may mimic the homozygous phenotype, *e.g.*, if the products of the remaining allele are capable of replacing structurally those of the missing allele. Thus, it is impossible to decide whether we are dealing with the phenocopy of a homozygote or with truly homozygous cells.

The possibility of mitotic crossing over was strengthened by the isolation of a group of variants from the MSWB sarcoma, which was originally induced in an (A × A.SW)F_1 hybrid. This tumor contains antigens D and K that are determined by the *H-2*^{a} allele, which has been introduced from strain A. In the parental A.SW strain, compatible variants can be readily isolated. These are characterized by the loss of both D and K. To isolate, if possible, variants that have lost only one of these antigens, host animals were produced by appropriate crosses that contained D but not K, and others that contained K but not D. In the hosts that were selected for the loss of K, variants were obtained that behaved in further transplantation tests as if they had lost K but contained D. Selection for the loss of D led to an unexpected result. The variants isolated showed invariably a transplantation behavior indicating the loss of both D and K. The interpretation of transplantation data in antigenic terms is always indirect. With this reservation, the findings suggested that K could be lost either alone or together with D, while the loss of D would always involve the loss of K. If mitotic crossing over is the mechanism at work, this would mean that K is located more distally than D in its relation to the centromere. Crossing over between D and K would then lead to the

loss of K without D, while crossing over between D and the centromere would always lead to the loss of both D and K. Since multiple crossovers do not occur at all, or only extremely rarely in known somatic crossover systems in *Drosophila* or in *Aspergillus* (*8*), loss of D without K would not be expected to occur with detectable frequency.

The possible occurrence of mitotic crossing over in mammalian somatic cells is of the greatest interest, as pointed out by Pontecorvo (*8*). If it would take place regularly, it would permit the genetic mapping of somatic cells, even in the absence of all other methods. While the data available now by no means prove that this is the mechanism, this lead must be followed up. As a first step, the previous transplantation data were supplemented with serologic analysis. In the present work, this analysis was made with quantitative absorption tests on some mouse sarcomas. The subsequent paper by Hellström describes a similar study on lymphomas with a direct cytotoxic test.

MATERIALS AND METHODS

Mice.—Mice of the following genotypes were used: A/Sn ($H\text{-}2^aH\text{-}2^a$), A.SW ($H\text{-}2^sH\text{-}2^s$), A.CA ($H\text{-}2^fH\text{-}2^f$), (A × A.SW)F_1 ($H\text{-}2^aH\text{-}2^s$), (A × A.CA)F_1 ($H\text{-}2^aH\text{-}2^f$), (A.SW × DBA/2)F_1 ($H\text{-}2^sH\text{-}2^d$), (A.SW × C3H)F_1 ($H\text{-}2^sH\text{-}2^k$), (A.CA × DBA/2)F_1 ($H\text{-}2^fH\text{-}2^d$), and (A.CA × C3H)F_1 ($H\text{-}2^fH\text{-}2^k$). The breeding nucleus of strains A/Sn, DBA/2, and C3H was maintained by a strict single-line brother × sister mating. To retain a high degree of homozygosis, the experimental animals were never permitted to deviate from the inbred nucleus by more than 2 brother × sister generations. Strains A.SW and A.CA, developed by Dr. George Snell, Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Maine, are coisogenic with A/Sn (*7*). They have a common strain A background but differ at the *H-2* locus. When received from Snell, the IR subline, A.SW, was in the 24th generation and A.CA in the 18th. They had been bred by brother × sister mating from the 12th generation. In our laboratory they were maintained by brother × sister mating, regularly interrupted by interpolated backcrossing to the A strain after each 3 or 5 brother × sister generations. The offspring of this backcross was intercrossed, and their progeny was subsequently challenged with tumors of A/Sn origin. The survivors were antigenically typed by the hemagglutination test of Gorer and Mikulska (*9*). Mice showing the *H-2* isoantigenic type of the IR subline entering the cross were subsequently used to replace the breeding pairs of this line. Their progeny was bred brother × sister for another 3 to 5 generations. This repeated backcrossing to the A/Sn strain was introduced to maintain, as nearly as possible, the identity of the common genetic background. At the time of these experiments, a total of 11 or 12 brother × sister crosses had been made since the mice were received from Dr. Snell. Backcrossing to the A/Sn strain preceded the first and the fifth brother × sister cross.

Previous studies involving skin and tumor grafting (*10*) revealed that A.SW and A.CA differed from strain A/Sn with regard to several weaker histocompatibility factors in addition to *H-2*—the origin of these differences has been traced by Snell to the early history of these lines (*11*). These weaker differences were easily and regularly transgressed by tumor grafts, but not by skin grafts.

All recipients of tumor grafts ranged from 18 to 25 g and were 3 to 4 months of age. They were kept on a standard pellet diet; drinking water and pellets were available *ad libitum*.

Tumors.—Sarcomas MSWB and MNSB were induced by methylcholanthrene in the thigh of (A × A.SW)F_1 mice in 1956. The transplantation behavior of these tumors, especially that of MSWB, was studied extensively in previous work (*1–5*). They were carried by serial transfer in the original genotype. In the present experiments, passages 3 to 22 of MSWB and 3 to 24 of MNSB were used.

Mammary cancer BSAB arose spontaneously in an (A × A.SW)F_1 female in 1958. For the present experiments, transplant generations 20 to 25 were used.

Sarcoma MACD was induced by methylcholanthrene in an (A × A.CA)F_1 mouse. It originated in 1957. For the present experiments, transfer generations 15 to 25 were used. It was always carried in (A × A.CA)F_1 mice.

Mammary carcinoma BACB arose spontaneously in an (A × A.CA)F_1 female. It was carried in the genotype of origin for 10 transfers.

The transplantation of tumor tissue was carried out after solid tumors were removed aseptically and freed from macroscopically visible necrotic areas. Immediately thereafter the tissue was pressed through a 60-mesh stainless-steel net into approximately 4 volumes of sterile Ringer's solution containing 100 IU penicillin per ml and 100 μg streptomycin per ml. The cell suspensions were inoculated subcutaneously into the right or left flank in volumes of 0.1 ml. The mice were examined twice a week for tumor development. Animals that remained negative or whose tumors regressed were kept under observation for a minimum of 3 months after inoculation. The designation "preimmunized mice" applies to animals whose inoculated tumors had regressed 2 or 3 weeks before actual testing.

Serologic methods.—Isoantiserums were produced by repeatedly inoculating A.SW and A.CA mice, respectively, with pooled cell suspensions derived from the spleen, liver, and kidney of strain A mice at intervals of 7 to 10 days. Six days after the fifth inoculation, the mice were bled from the retro-orbital sinus through a glass capillary. Blood, pooled from several mice, was allowed to clot at room temperature. The pooled serums were kept at −20° C. The serums were tested against C3H and DBA erythrocytes by the human-serum dextran hemagglutination technique (*9*).

Irradiation.—X rays were generated in a Siemens X-ray machine at 185 kv and 15 ma and were filtered by 1 mm Al.

Absorption.—Tumor or lymph-node tissue was cut into small pieces with scissors and washed once with Ringer's solution. The minced tissue was transferred into a small Erlenmeyer flask containing 2 to 3 volumes of 0.25 percent trypsin solution. It was stirred for 15 to 20 minutes with a magnetic stirrer. The supernatant fluid was collected and stored in an ice bath. New trypsin solution was added to the remaining tissue, and stirred for another 15 to 20 minutes. The supernatant was pooled with the stored material. This procedure was repeated 4 to 5 times until about 10^8 dispersed cells were obtained. According to the method of Schrek (*12*), the approximate concentration of viable cells was determined by counting in a Burker hemocytometer after dilution with Tyrode's solution containing 0.5 percent eosin. Usually only a minor fraction (about 10%) of the cells was stained with eosin. Volumes containing a predetermined cell number were centrifuged for 10 minutes; the cells were washed with 2 percent dextran (Intradex Salt Free, Glaxo, Ltd., London; 6%, diluted with saline) and recentrifuged. Care was taken during this procedure to recover the cell material quantitatively. The antiserum was diluted 1:20 with dextran, and 0.1 ml was added to each tube containing the packed cells and 3 small glass beads. The tubes were placed on a mechanical vibrator, and the contents were agitated for 60 minutes at room temperature. During this time, the cells distributed uniformly as single-cell suspensions with only occasional clumps. Subsequently the tubes were centrifuged, and the serum was collected and stored overnight at −20° C. The following day it was tested against C3H and DBA/2 erythrocytes.

RESULTS

Isolation of Variants

As outlined in the introduction, the purpose of this work was to investigate the possible segregation of antigens D and K of the *H-2* system during the formation of variant sublines from different tumors of F_1-hybrid origin. The tumors had been either derived from (A × A.SW)F_1 hybrids (*H-2^{a}H-2^s*) or from (A × A.CA)F_1 (*H-2^{a}H-2^f*). Antigens D and K were introduced from the A parent in both crosses. Table 1 shows the antigenic formula of the genotypes used.

Variants were isolated in selective hosts of an F_1-hybrid type differing from the genotype in which the tumor originated by the A strain having been replaced in each outcross either by DBA or by C3H. DBA contains D but lacks K, while C3H contains K but lacks D. A complete list of the known antigenic composition of the *H-2* system of the selective hosts is given in table 1.

As a rule, the following procedure was used to isolate variants:

The primary tumor was transplanted serially through animals isologous with the original host genotype. One or several tubes containing pieces of the original neoplasm were preserved in the frozen storage by the procedure

TABLE 1.—*H-2* genotype and antigenic constitution of the original and the selective tumor hosts

Host	Genotype	Known antigenic composition of the *H-2* system*	Note
Original:			
(A × A.SW)F_1	*H-2^{a}H-2^s*	$\frac{\text{ACDEFHJKMNY}}{\text{CEFGS}}$	Both D and K present
Selective:			
(DBA × A.SW)F_1	*H-2^{d}H-2^s*	$\frac{\text{CDE}^d\text{FHJMN}}{\text{CEFGS}}$	D present, K absent
(C3H × A.SW)F_1	*H-2^{k}H-2^s*	$\frac{\text{ACEHKY}}{\text{CEFGS}}$	K present, D absent
A.SW	*H-2^{s}H-2^s*	CEFGS	Both D and K absent
Original:			
(A × A.CA)F_1	*H-2^{a}H-2^f*	$\frac{\text{ACDEFHJKMNY}}{\text{GHI}}$	Both D and K present
Selective:			
(DBA × A.CA)F_1	*H-2^{d}H-2^f*	$\frac{\text{CDE}^d\text{FJHMN}}{\text{GHI}}$	D present, K absent
(C3H × A.CA)F_1	*H-2^{k}H-2^f*	$\frac{\text{ACEHKY}}{\text{GHI}}$	K present, D absent
A.CA	*H-2^{f}H-2^f*	GHI	Both D and K absent

* According to Amos (*13*).

described previously (*14*). At regular intervals, the transplanted tumor line was "cut back" to the earliest available transfer generations by thawing available tubes to avoid the use of lines with excessively long transplantation histories. No lines were used beyond their 25th-transfer generation.

The tumors grew in 100 percent, or nearly 100 percent, of isologous recipients, while they usually regressed in most, if not all, selective hosts. Whenever progressive growth was obtained in a selective host [*e.g.*, in (A.SW × DBA)F_1], the outgrowing tumor was tested further to characterize its transplantation behavior. It was implanted into a group of mice isologous with the selective host in which it appeared [(A.SW × DBA)F_1] as well as into groups of the other selective hosts [in this case, (A.SW × C3H)F_1 and A.SW]. Whenever the mouse supply permitted it, the tumor was also tested in selective hosts preimmunized against tissues of strain A origin. At the same time, it was also implanted into strain A mice and into mice of other coisogenic lines carrying foreign alleles at the *H-2* locus, such as A.CA and A.BY.

The term "variant subline" will be used to designate all lines that were clearly compatible with one or several selective hosts, but not with strain A mice or with foreign genotypes. Sometimes several passages were necessary in the selective host before the variant lines became stabilized (*4*). Variants specifically compatible with well-defined host genotypes

could be clearly distinguished from nonspecific forms that grew indiscriminately in foreign genotypes. Nonspecific lines probably arise by other mechanisms than specific variant sublines (*15*).

A summary of all transplantation tests, performed until the time of this writing, is given in table 2. Five tumors were studied and a total of 33 variant sublines was tested. It appears that all variants selected from hybrids with a DBA component were compatible with the same type of hybrid. In addition, some of them were also compatible with the C3H hybrid, while others were not. On the other hand, variants selected from the C3H hybrids were in all but one case compatible with both the DBA and the C3H hybrids. One variant selected in the C3H hybrid was *not* breeding true (*i.e.*, did not grow upon further transfer) in C3H hybrids but did grow well in DBA hybrids. No variants selected in the C3H hybrid were compatible with the C3H hybrid alone, to the exclusion of the DBA hybrid. Whenever tested, variants compatible with the C3H and the DBA hybrid were also compatible with the parental strain itself entering the selective cross, that is, A.SW in the $(A \times A.SW)F_1$ tumors and A.CA in the $(A \times A.CA)F_1$ tumors.

The reaction of the DBA hybrids is chiefly directed against the K component of the tumor cells, while the C3H hybrids react against the D component (*13*). The parental strain itself entering the cross (A.SW and A.CA, respectively) reacts against both D and K. It was therefore expected that the DBA hybrids would have favored variants that had lost K, the C3H hybrids would have favored variants that lost D, while the parental strain in question would have favored variants that lost both components. Previous serologic studies on variants selected from the MSWB sarcoma compatible with the parental A.SW strain have confirmed the expectation that both D and K are actually lost in the latter (*3*).

The asymmetrical results of the transplantation tests summarized in table 2 are in full agreement with previous studies on a small sample of MSWB variants. They suggest that variant cells can arise that have lost K alone or both D and K and that such variants can be concentrated in appropriate selective hosts. On the other hand, no variants could be detected where the transplantation results would have suggested that they had lost D but still contained K. Before this finding is further discussed, it is necessary to verify these tentative interpretations of the transplantation tests in terms of isoantigens by direct serologic analysis. In this study the antigenic composition of representative tumors and their variants was examined by quantitative absorption tests *in vitro*.

Absorption Tests

Contribution of host cells.—Since the tests to be described were based on the absorption of preformed isoantibodies by tumor-cell suspensions grown in hosts of different, known genotypes, it was essential to investigate whether and to what extent the presence of host cells containing antigens absent from the tumor cells could influence the results.

TABLE 2.—Summary of transplantation behavior of variant sublines

Tumor	Original genotype	Selective host	Compatibility*		
			A.SW × DBA + A.SW × C3H −	A.SW × DBA + A.SW × C3H +	A.SW × DBA − A.SW × C3H +
MSWB sarcoma	(A × A.SW)F_1	(A.SW × DBA)F_1	2/6	4/6	0/6
		(A.SW × C3H)F_1	1/9	8/9	0/9
MNSB sarcoma	(A × A.SW)F_1	(A.SW × DBA)F_1	3/4	1/4	0/4
BSAB carcinoma	(A × A.SW)F_1	(A.SW × DBA)F_1	1/3	2/3	0/3
		(A.SW × C3H)F_1	0/3	3/3	0/3
			A.CA × DBA + A.CA × C3H −	A.CA × DBA + A.CA × C3H +	A.SW × DBA − A.CA × C3H +
MACD sarcoma	(A × A.CA)F_1	(A.CA × DBA)F_1	2/4	2/4	0/4
		(A.CA × C3H)F_1	0/3	3/3	0/3
BACB carcinoma	(A × A.CA)F_1	(A.CA × C3H)F_1	0/1	1/1	0/1

*The figures denote the number of variant sublines compatible with (*i.e.*, capable of progressive growth in nearly 100%) the genotype designated + in the table heading, but incompatible with (*i.e.*, regularly regressing in) the genotype designated −, over the total number of variant sublines tested.

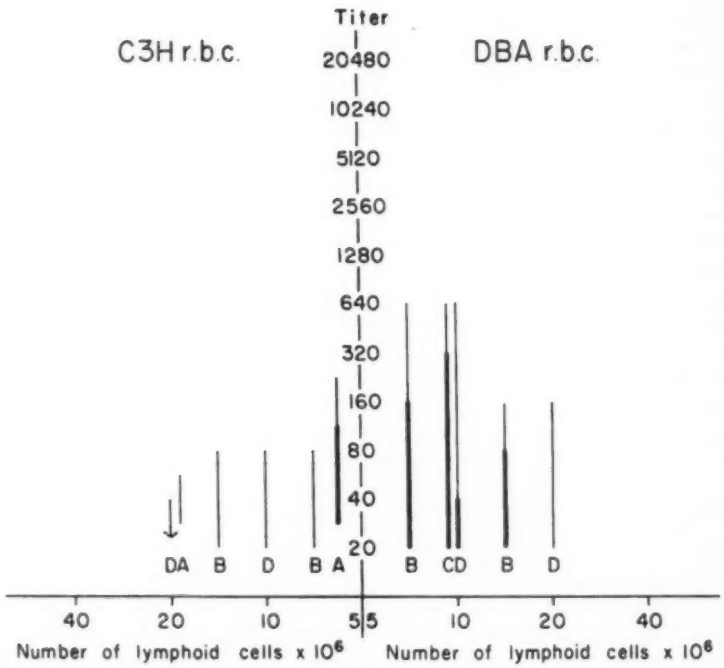
In a preliminary experiment, normal (A × A.SW)F_1 hybrid lymph-node cells were tested for their ability to absorb anti-K and anti-D antibodies. For this and all subsequent absorption tests the same isoantiserum was used. It has been produced by immunizing A.SW mice with strain A cells. As far as the known major components of the *H-2* system are concerned, the reaction of this serum against C3H cells is essentially directed against the K component, while DBA cells will mainly react by virtue of their D antigen. Absorption of the anti-DBA activity will therefore indicate the presence of antigen D in the absorbing tissue, while the removal of anti-C3H activity will indicate the presence of K. Cross reactivity between C3H and DBA red cells was absent. A.SW, anti-C3H serum highly active against C3H cells, did not agglutinate DBA cells. Absorption of the isoantiserum with cells containing antigens of DBA origin did not remove anti-C3H activity.

When tested for hemagglutinating activity, the A.SW anti-A serum used for all experiments had a titer of 1:20, 480 against both C3H and DBA red cells. When the serum was incubated with different numbers of (A × A.SW)F_1 lymph-node cells for 1 hour at room temperature, the activity was absorbed as shown in text-figure 1.

Both the anti-D and the anti-K activities were absorbed by the lymphocytes as expected.

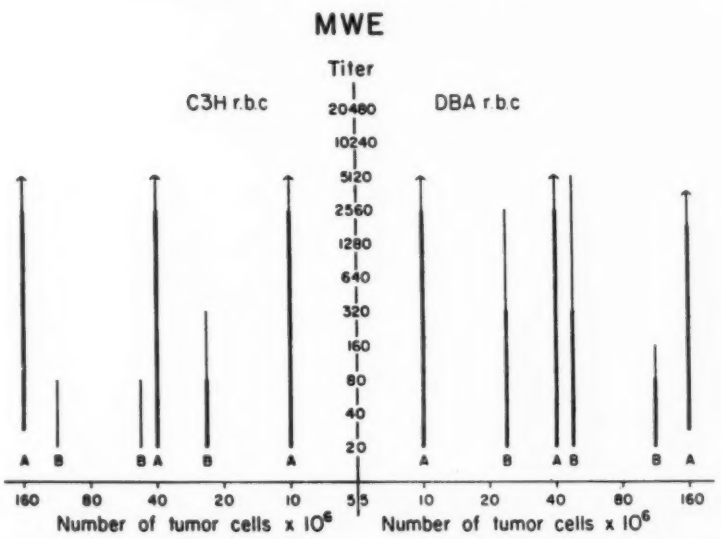
To study the possible contribution of stroma and inflammatory cells to the absorption pattern exhibited by a transplanted tumor, experiments were carried out with the MWE sarcoma, originally induced by methylcholanthrene in an A.SW host. The tumor was transplanted to one group of A.SW mice and to another group of (A × A.SW)F_1 hybrids. While the contribution of A.SW host cells cannot influence the absorption behavior of the tumor in the present test, serious admixture of (A × A.SW)F_1 hybrid host cells would be of considerable importance, since they would absorb anti-D and anti-K activity as well. Text-figure 2 shows the results.

The contribution of the host cells was clearly detectable. Since the variant sublines tested were usually carried in the F_1 hybrid host of origin and these hosts carried the antigens, the possible loss of which from the tumor cells was to be investigated, it appeared necessary to avoid this potential source of error by the inoculation of the tumors into hosts that did not carry the antigens in question. One case in point is shown in text-figure 3. One variant of the MSWB sarcoma [originally induced in an (A × A.SW)F_1 hybrid] has lost both D and K as will be shown. Denoted MSWB-1089, this variant is routinely propagated in (A × A.SW)F_1 hybrids. The reason for carrying it in the genotype of origin is to test whether variant formation can be reversible. This has, incidentally, given entirely negative results so far. The missing antigenic phenotype was never re-established. As shown in text-figure 3, the variant absorbed both anti-D and anti-K activities when taken from the F_1 hybrid host. After inoculation to homozygous adult A.SW hosts, where the tumor grew progressively due to its loss of D and K, its absorbing capacity



TEXT-FIGURE 1.—Absorption of A.SW-anti-A serum with (A × A.SW)F_1 lymph-node cells. The unabsorbed serum had a titer of 1:20, 480 against both C3H and DBA red cells. *Left half* of the chart shows the titer against C3H erythrocytes after the serum was incubated for 1 hour at room temperature with different numbers of cells. *Right half* shows the titers of the same serums against DBA erythrocytes. Hemagglutination was scored as +++, ++, +, ±. In all charts, +++ and ++ are marked with a heavy line, + and ± with thin line. *Arrows*, pointing upward, indicate the highest serum dilution tested that still gave a positive reaction; these samples were not titrated to endpoint. *Arrows*, pointing downward, indicate the lowest dilution tested that gave a negative reaction. Each capital letter denotes a different experiment.

vanished. Another possible source of error enters the picture here, however. If it is assumed that the tumor cells still contain D and/or K, it would be expected that the A.SW host forms antibodies against them. If these antibodies coat the cell surface to an appropriate extent, they might conceivably counteract the absorbing capacity of the tumor cells in the absorption tests *in vitro*. To avoid both sources of error, it seemed necessary to carry out absorption tests with tumor cells that have been taken from hosts that neither carry the antigen, the possible loss of which is to be investigated, nor form antibodies against them if they are present. This was accomplished by inoculating all tumors of (A × A.SW)F_1 origin into newborn A.SW mice and all tumors of (A × A.CA)F_1 origin into newborn A.CA mice. All newborn hosts were radiated with 400 r within a few hours after birth and immediately thereafter inoculated with

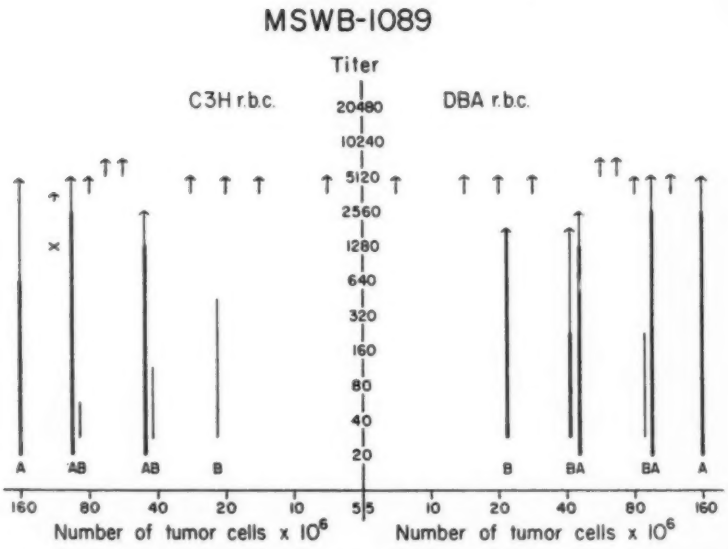


TEXT-FIGURE 2.—Absorption of A.SW-anti-A serum with MWE sarcoma cells (A.SW origin), taken from A.SW (cols. A) or (A × A.SW)F_1 hosts (cols. B), respectively. Experimental details identical with those explained in legend of text-figure 1.

the tumor. Since such hosts lack antigens D and K themselves and their antibody-forming ability is impaired, they fulfill both requirements. Text-figure 3 shows that variant MSWB-1089 failed to absorb anti-D and anti-K activities even in this case, in complete agreement with the postulated loss of D and K from the tumor cells.

Analysis of variants.—The original line of the MSWB sarcoma has been carried in (A × A.SW)F_1 hybrids during 22 serial transfers. The summary of the transplantation results and of typical absorption tests appears in text-figure 4. The sarcoma cells are able to absorb both anti-C3H and anti-DBA activity, which indicates that they contain K and D antigens. The number of cells necessary to absorb serum activity against DBA erythrocytes was at least twice the number required to absorb anti-C3H activity to the same degree, which shows that the cells contain more K than D. The presence of both K and D on the MSWB sarcoma cells was already demonstrated in previous studies that involved testing the tumor cells for their ability to provoke the formation of hemagglutinins in foreign strains (*3*). However, in such tests it could not be decided with certainty to what extent the stroma cells of the (A × A.SW)F_1 hybrid host may complicate the results since they are always present in the immunizing inoculum.

In the present work, MSWB sarcoma cells have been tested for their absorbing potentialities both after growth in (A × A.SW)F_1 hybrids (expts. B and C in text-fig. 4) and after passage through newborn, irradiated A.SW mice that did not carry the antigens in question (expts. A, D,

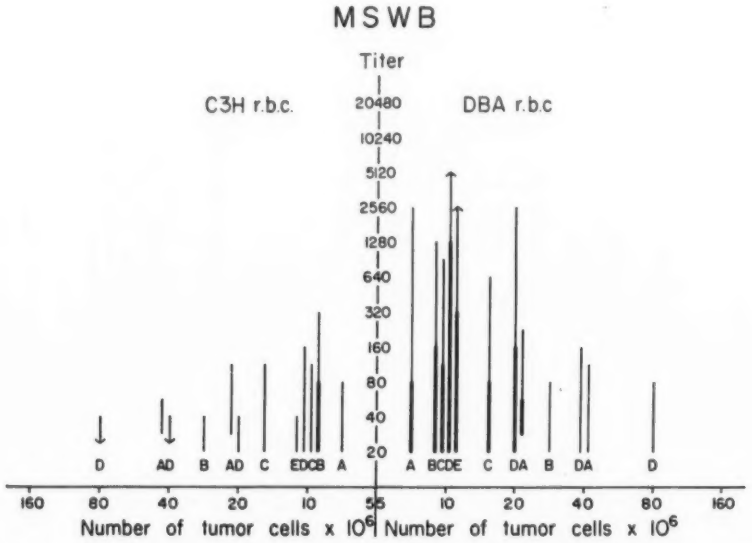


TEXT-FIGURE 3.—Absorption of A.SW-anti-A serum with variant subline MSWB-1089. The tumor was grown in adult A.SW (cols. A) and in adult (A × A.SW)F_1 hosts (cols. B), respectively. In addition, as indicated by the isolated arrows, the absorption tests were made with the same tumor line grown in irradiated newborn A.SW mice. Experimental details identical with those explained in legend of text-figure 1.

and E in text-fig. 4). Essentially the same results have been obtained with both types of material, and it seems, therefore, safe to conclude that D and K are present in the sarcoma cells.

Text-figure 5 illustrates the derivation of the variant MSWB sublines used for the absorption tests. From a tumor that grew in an (A.SW × DBA)F_1 mouse after the inoculation of an MSWB cell suspension, a variant subline was established that was carried in (A.SW × DBA)F_1 mice for 7 passages. Subsequently, it was returned to the original (A × A.SW)F_1 genotype. This subline was designated MSWB-1032. It was fully compatible with (A.SW × DBA)F_1 hybrids but not with (A.SW × C3H)F_1 or with A.SW. In (A.SW × C3H)F_1 hybrids, progressively growing tumors were obtained in only 2 of 29 recipients. From one (A.SW × C3H)F_1, a second subline was derived. After being carried for 2 more passages in (A.SW × C3H)F_1 and tested on each transfer in preimmunized (A.SW × C3H)F_1 hosts (*cf.* text-fig. 5), it was returned into (A × A.SW)F_1 hybrids. Designated MSWB-1089, this subline remained fully compatible with (A.SW × C3H)F_1 mice even after a long series of passages in the original (A × A.SW)F_1 genotype.

A third tumor subline, designated MSWB-1090, was derived from the same (A.SW × C3H)F_1 host as MSWB–1089, but it was immediately

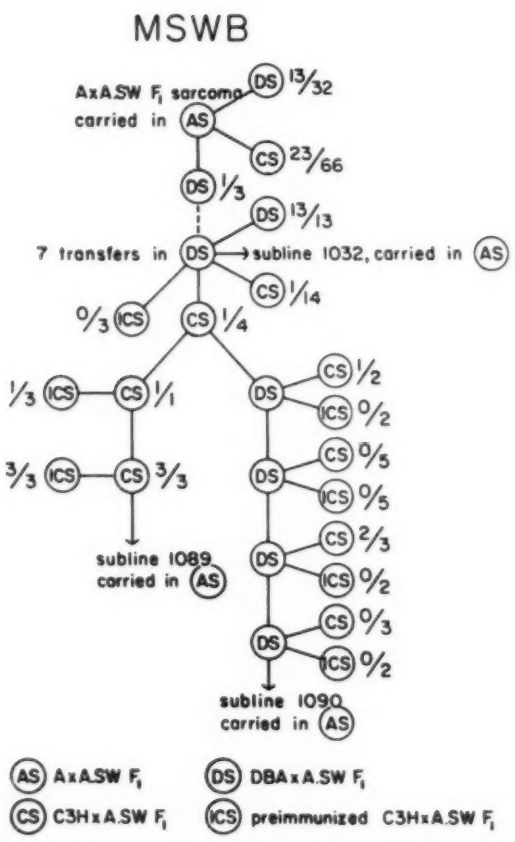


TEXT-FIGURE 4.—Absorption of A.SW-anti-A serum with MSWB sarcoma cells [of (A × A.SW)F_1 origin]. Experimental details as described in legend of text-figure 1.

Summary of transplantation tests with MSWB

Range of passages in (A × A.SW)F_1	(A × A.SW)F_1	A	A.SW	(A.SW × DBA)F_1	(A.SW × C3H)F_1
3–22	48/48	2/22	14/34	8/21	4/16

The figures denote the number of mice killed by progressively growing tumors over the total number inoculated.

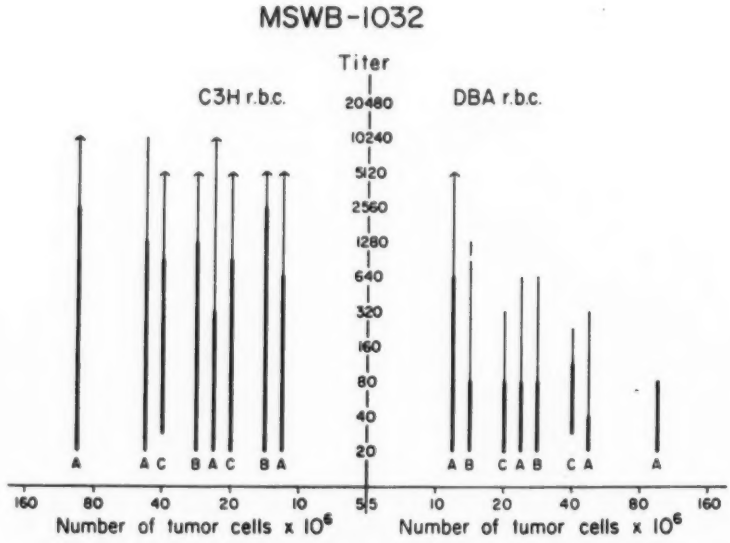


TEXT-FIGURE 5.—Transplantation history of variant MSWB sublines. The data concerning the growth of the original MSWB line in (A.SW × DBA)F_1 and (A.SW × C3H)F_1 hybrids represent pooled results of all transplantation tests performed with a number of different parallel sublines started from frozen samples of the earliest passages of this tumor at different times.

returned to and carried in (A.SW × DBA)F_1 mice for 4 passages (text-fig. 5). Subsequently, it was carried in (A × A.SW)F_1. This line was compatible with (A.SW × DBA)F_1, but not with (A.SW × C3H)F_1. All 3 variant sublines were thus carried in the original, nonselective genotype, partly because it was considered necessary to have a common host for maintenance and partly, as mentioned before, to study the possible reversibility of the changed serologic and transplantation behavior of the variant sublines.

Text-figure 6 shows typical absorption experiments performed with the MSWB-1032 line and summarizes its transplantation behavior.

It appears that 10^8 variant cells did not remove any activity against C3H erythrocytes to any detectable degree, while absorption with 10^7 cells



TEXT-FIGURE 6.—Absorption of A.SW-anti-A serum with subline MSWB-1032. Experimental details as described in legend of text-figure 1.

Summary of transplantation tests with MSWB-1032

Number of passages in	Host genotypes				
$(A \times A.SW)F_1$	$(A \times A.SW)F_1$	A	A.SW	$(A.SW \times DBA)F_1$	$(A.SW \times C3H)F_1$
8	18/18	0/8	3/14	28/28	2/29

The figures denote the number of mice killed by progressively growing tumors over the total number inoculated.

of the original type (MSWB) resulted in a well-pronounced decrease of the agglutinating titer (*cf.* text-fig. 4). The activity of the serum against DBA red blood cells decreased to the same extent as with MSWB. This indicates that this variant has lost antigen K but still contains antigen D. This is in good agreement with its transplantation behavior since it grew in (A.SW × DBA)F_1 in 100 percent but grew only occasionally in (A.SW × C3H)F_1 or in A.SW. This behavior was stable and unchanged after 8 passages in (A × A.SW)F_1.

Essentially identical results were obtained with subline MSWB-1090 (text-fig. 7). The serologic and transplantation behavior of this line was unchanged after 11 passages in (A × A.SW)F_1 hybrids.

Text-figure 8 shows the results with subline MSWB-1089. Both K and D appear to be lost. This is in good agreement with the transplantation behavior of this line since it was fully compatible with (A.SW × DBA)F_1, (A.SW × C3H)F_1, and A.SW mice. The behavior of this line did not change after 8 serial passages in (A × A.SW)F_1 hybrids. It may be pointed out that line MSWB-1089 had been selected in two steps. It had been derived from subline MSWB-1032 that lost K but not D. After subsequent selective transfer through (A.SW × C3H)F_1, D was also lost.

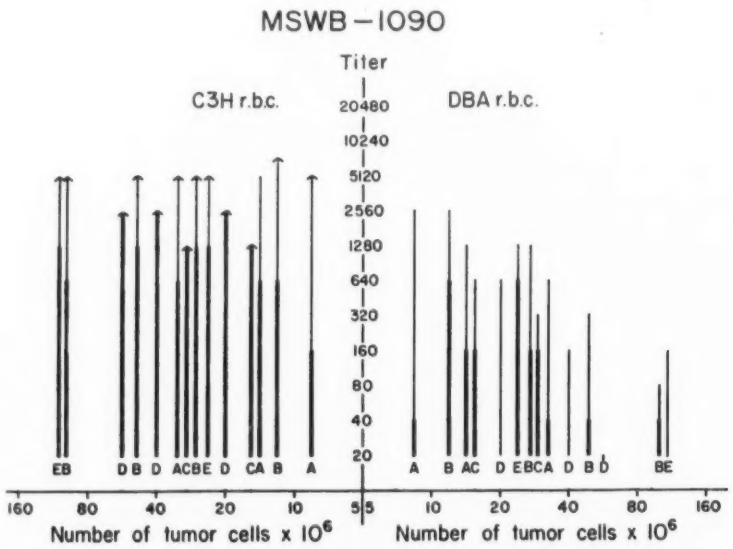
Serologic and transplantation results similar to those previously exemplified were obtained with other representative tumors and their sublines listed in table 1. With established variant sublines, there was always a good agreement between the transplantation results and the absorption experiments. Text-figure 9 shows the results obtained with another (A × A.SW)F_1 sarcoma MNSB and its variant subline, MNSB-17D, derived from an (A.SW × DBA)F_1 hybrid inoculated simultaneously with the 17th passage of MNSB.

As indicated by the transplantation results and the absorption data, MNSB-17D has lost K but not D, while the original tumor line contains both.

Text-figure 10 shows the results obtained with a sarcoma of the (A × A.CA)F_1 genotype (MACD) and its variant subline MACD-25D. Although selected in (A.CA × DBA)F_1, the variant appears to have lost both D and K.

DISCUSSION

The main antigens involved in this study are D and K. Nevertheless other antigens shared by A and C3H or DBA are also known. They influence, most probably, the transplantation behavior and contribute to the results of the serologic tests. Accordingly, one has to assume that the results obtained in the present work reflect the behavior of groups of antigens, determined by closely linked genes, rather than only D and K. Gorer (*16*) presented circumstantial evidence that the *H-2*^{a} locus of strain A arose by meiotic crossing over of the *H-2*^{d} and *H-2*^{k} loci. There are no antigens known to be present in *H-2*^{a} that are not also present in

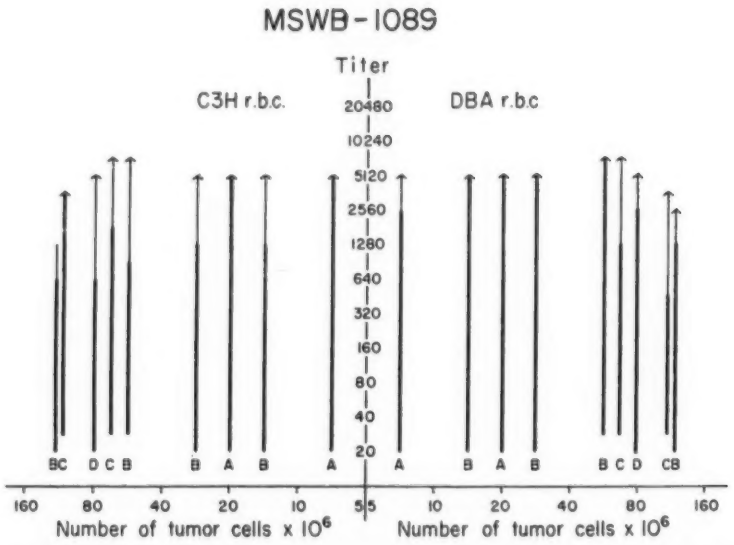


TEXT-FIGURE 7.—Absorption of A.SW-anti-A serum with subline MSWB-1090. Experimental details as described in legend of text-figure 1.

Summary of transplantation tests with MSWB–1090

Number of passages in	Host genotypes						
$(A \times A.SW)F_1$	$(A \times A.SW)F_1$	A	A.SW	$(A.SW \times DBA)F_1$	$(A.SW \times C3H)F_1$	$(A.SW \times DBA)F_1$ preimmunized against A	$(A.SW \times C3H)F_1$ preimmunized against A
11	24/24	0/14	6/16	38/38	1/18	13/13	0/20

The figures denote the number of mice killed by progressively growing tumors over the total number inoculated.



TEXT-FIGURE 8.—Absorption of A.SW anti-A serum with MSWB-1089 sarcoma cells. Experimental details as described in legend of text-figure 1.

Summary of transplantation tests with MSWB–1089

	Host genotypes						
Number of passages in (A × A.SW)F_1	(A × A.SW)F_1	A	A.SW	(A.SW × DBA)F_1	(A.SW × C3H)F_1	(A.SW × DBA)F_1 preimmunized against A	(A.SW × C3H)F_1 preimmunized against A
8	18/18	0/4	10/10	21/21	25/25	4/4	5/5

The figures denote the number of mice killed by progressively growing tumors over the total number inoculated.

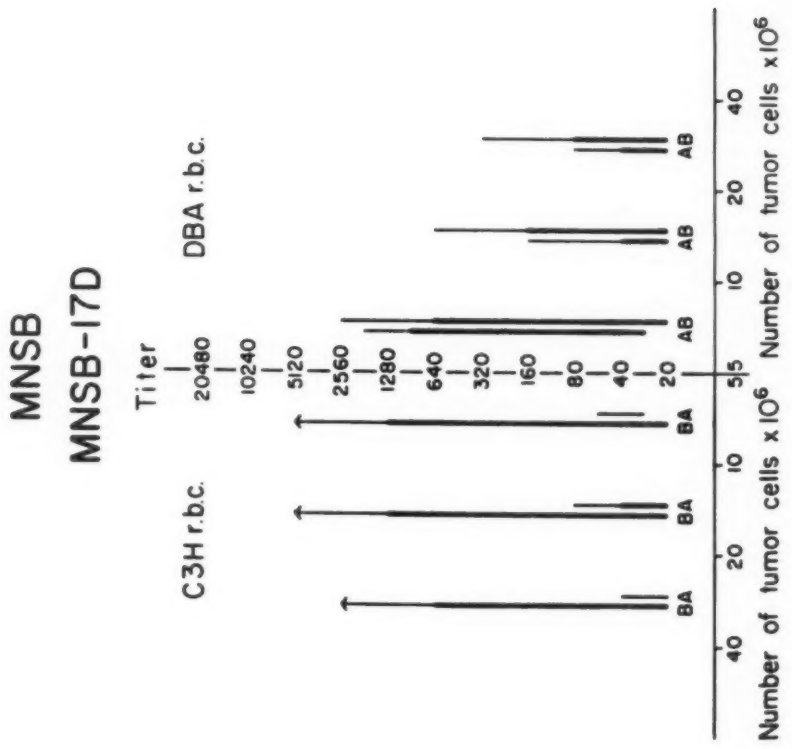
H-2^d or *H-2*^{k}. Therefore, it is probable that those antigens of *H-2*^{a} that are shared with *H-2*^{d} belong to one group, and those shared with *H-2*^{k} to another closely linked group. It is the behavior of these groups that is probably represented by what has been referred to as antigens D and K, for the sake of simplicity in this work.

The transplantation studies on 5 tumors in the present work fully confirm and extend the preliminary findings on 1 sarcoma (*3*, *4*) in showing that heterozygous tumors containing antigens D and K of the *H-2* system can give rise to variants that have either lost K alone or both D and K. Although appropriate selective hosts were available, it was not possible to isolate variants that have lost D while still containing K. The interpretation of transplantation tests in antigenic terms remains tentative, however, until confirmed by serologic analysis. In the present study, such an analysis, based on a quantitative absorption test, fully confirmed the interpretation of the transplantation results. To obtain unbiased serologic data, it was important to exclude possible antigenic contribution of the tumor stroma and to avoid antibody coating of the cell surface. The ideal host does not contain the antigens, the presence or absence of which in the tumor cells is to be investigated; neither can it form antibodies against them. Newborn, whole-body irradiated mice of one parental strain were found suitable for this purpose.

The asymmetrical behavior of D and K is quite puzzling. One possible interpretation would be that D is a precursor of K. *H-2* conbinations are known, however, that lack D but still contain K, such as *H-2*^{k} and *H-2*^{m}. Another alternative explanation, used as our working hypothesis, is mitotic crossing over, also suggested by the findings mentioned in the introduction. Mitotic or somatic crossing over has been discovered by Stern (*17*) in *Drosophila* in 1936. Stern's observation was based on the occurrence of "twin spots," where, on *Ab/aB* individuals, the recessive phenotype *aa* appeared in an area adjacent to one with the recessive phenotype *bb*. Stern's interpretation was that mitotic crossing over has occurred between 2 of the 4 chromatids of the relevant homologous chromosomes. It was localized between the B locus and the centromere, and the next mitosis led to sister cells with the genotypes *aB/aB* and *Ab/Ab*.

Somatic crossing over has received its most important application so far in the work of Pontecorvo and his collaborators (*8*) who demonstrated its usefulness as a tool for genetic analysis. Their procedure was essentially based on the selection of segregants from heterozygous diploid strains of *Aspergillus* that were homozygous or hemizygous for certain distal markers and subsequent analysis of these segregants for their residual genotypes. This analysis has in no way been inferior to ordinary genetic mapping based on meiotic crossing over.

Pontecorvo has emphasized repeatedly the possible applicability of this procedure to tissue cells of higher organisms. This possibility has been often dismissed previously since it was assumed that somatic pairing of homologous chromosomes is an absolute prerequisite. While somatic

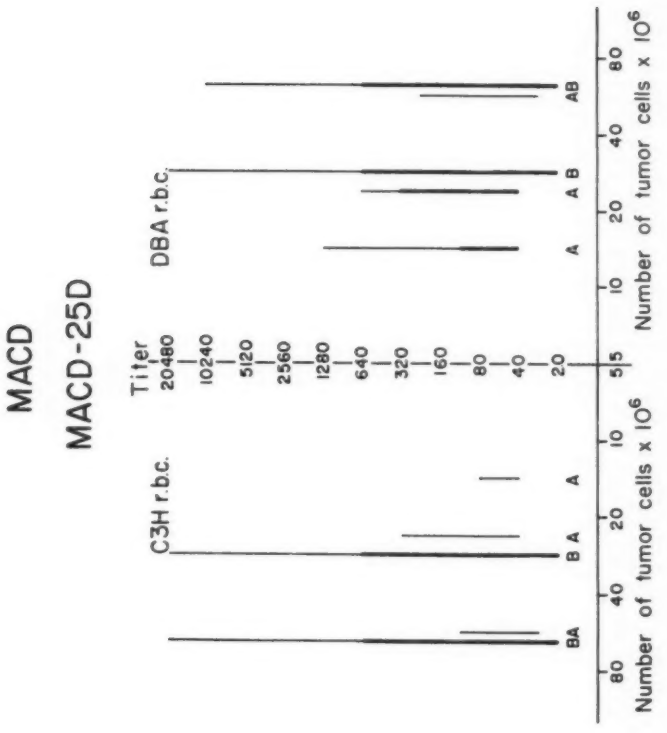

MNSB
MNSB-17D
Titer
C3H r.b.c.
DBA r.b.c.
20480
10240
5120
2560
1280
640
320
160
80
40
20
5|5
BA
BA
BA
AB
AB
AB
40 20 10 Number of tumor cells x10⁶
Number of tumor cells x10⁶ 10 20 40

TEXT-FIGURE 9.—Absorption of A.SW-anti-A serum with tumor MNSB (cols. A) and its subline, MNSB-17D (cols. B). Experimental details as described in legend of text-figure 1.

Summary of transplantation tests

Range of passages	Host genotypes: $(A \times A.SW)F_1$	A	A.SW	$(A.SW \times DBA)F_1$	$(A.SW \times C3H)F_1$	$(A.SW \times DBA)F_1$ preimmunized against A
			MNSB			
in $(A \times A.SW)F_1$ 3–24	42/42	0/10	1/30	3/12	0/8	
			MNSB-17D			
in $(A.SW \times DBA)F_1$ 5	2/2	0/5	0/11	10/10	1/8	3/3

The figures denote the number of mice killed by progressively growing tumors over the total number inoculated.


MACD
MACD-25D
Titer
C3H r.b.c.
DBA r.b.c.
20480
10240
5120
2560
1280
640
320
160
80
40
20
5/5
BA
B A
A
A
A B
AB
80 40 20 10
Number of tumor cells x 10⁶

TEXT-FIGURE 10.—Absorption of A.CA-anti-A serum [titer 1 : 20, 480 with both C3H and DBA erythrocytes with tumor MACD (cols. A) and its subline, MACD-25D (cols. D)]. Experimental details as described in legend of text-figure 1.

Summary of transplantation tests

Range of passages	Host genotypes					
	$(A \times A.CA)F_1$	A	A.CA	$(A.CA \times DBA)F_1$	$(A.CA \times C3H)F_1$	$(A.CA \times DBA)F_1$ preimmunized against A
			MACD			
in $(A \times A.CA)F_1$ 15–25	22/22	0/18	7/20	6/12	4/8	
			MACD-25D			
in $(A.CA \times DBA)F_1$ 3			8/8	6/6	4/4	2/2

The figures denote the number of mice killed by progressively growing tumors over the total number inoculated.

pairing is a regular event in *Drosophila*, butterflies, and moths, and less regular in other lower organisms, it has been demonstrated only exceptionally in higher organisms (*18*). However, the validity of this requirement has been seriously questioned. As emphasized by Stern (*19*), we are so ignorant about the mechanism of crossing over that negative cytological evidence regarding the absence of somatic pairing at metaphase can hardly be taken as a serious objection against the possible occurrence of somatic crossing over. Similar views have been put forward by Pontecorvo (*8*).

The interpretation of the present phenomenon as mitotic crossing over would imply that K is located more distally in relation to the centromere than D. Crossing over between D and K would then give rise to variants that have lost K but not D, while crossing over between the centromere and D would give rise to variants that have lost both D and K. It would also have to be implied that D and K must be at quite some distance from each other. This is not unlikely in view of the finding that meiotic crossovers involving D and K occur as often as 1 percent (*16*).

In our continued work, attempts will be made to extend the investigation to other antigens of the *H-2* system to see whether they can be arranged in a consecutive linear order in relation to D and K. Further work on D and K will involve more intensive efforts to isolate the missing variant containing K but lacking D. If found, it would invalidate the crossing-over hypothesis.

Attempts will also be made to extend this study to other chromosomes. The first linkage group of the mouse, containing the isoantigenic determinants *H-1* and *H-4*, is now well marked in suitable coisogenic resistant lines (*20*) and appears to be an outstanding candidate for experiments of this type.

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Studies on the Mechanism of Isoantigenic Variant Formation in Heterozygous Mouse Tumors. II. Behavior of *H-2* Antigens D and K: Cytotoxic Tests on Mouse Lymphomas [1, 2]

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SUMMARY

Two (A × A.SW)F_1 hybrid mouse lymphomas were tested for transplantability to (A.SW × DBA)F_1, (A.SW × C3H)F_1, and A.SW mice. Tumors arising in these mice were tested for the presence of the *H-2*-determined isoantigens D and K, by the cytotoxic technique of Gorer and O'Gorman. Each of the 12 tumors which grew progressively in (A.SW × DBA)F_1 mice had lost antigen K, while D was present in varying degrees in 11 of 12 tumors. Six of 6 takes in (A.SW × C3H)F_1 mice had lost both antigens D and K. In three instances, the absence of D in a significant proportion of the cells in A.SW × DBA sublines could be correlated with transplantability to (A.SW × C3H)F_1 and A.SW mice, where the cells lacking D were apparently selected for. The fact that antigen K can be lost alone or together with D, but D cannot be lost without K is tentatively attributed to somatic crossing over.—J. Nat. Cancer Inst. 27: 1095–1105, 1961.

AS DESCRIBED in the preceding paper (*1*), sarcomas of heterozygous F_1 hybrid origin, containing the antigens D and K of the *H-2* system in a single dose, could give rise to variant sublines in appropriate selective hosts that had lost either K alone or D and K. No variants could be found having lost D alone, without K. For reasons discussed in the previous paper, this was tentatively attributed to somatic crossing over. This is a mechanism that has also been suggested by the phenotypic similarities between homozygous tumors and parental-strain variants, isolated from F_1 hybrid tumors, in their sensitivity to *H-2* isoantiserums *in vitro* and ability for nonspecific growth in foreign genotypes (*2*, *3*).

This paper presents a study with lymphoma cells, with results analogous to those described in the preceding paper (*1*). Methodologically, lymphoma cells offer special advantages for this type of study, since they are eminently sensitive to cytotoxic humoral antibodies, which permit antigenic analysis at the cellular level with the cytotoxic test of Gorer and O'Gorman (*4*, *5*).

[1] Received for publication May 22, 1961.

[2] This work was supported by grants from the Swedish Medical Research Council and the Swedish Cancer Society, and by grant C-3700 to Professor G. Klein from the National Cancer Institute, National Institutes of Health, Public Health Service.

[3] My sincere thanks to Professor G. Klein and Dr. E. Klein for their valuable advice and to Mrs. Anna-Stina Höljer and Mrs. Gunnel Isakson for valuable technical assistance.

MATERIALS AND METHODS

The mouse strains used and the methods for tumor transplantation have been described in the preceding paper (*1*).

Tumors.—Lymphoma LNSF originated in an (A × A.SW)F_1 female, inoculated subcutaneously in the right flank 2 months previously with HNSC, a thyrotropic pituitary tumor. While there was no detectable growth at the site of inoculation of the pituitary tumor, the axillary, inguinal, and mesenterial lymph nodes and spleen were greatly enlarged. Pooled cell suspensions of lymph nodes and spleen were transplanted to isologous recipients and gave rise to local, progressively growing tumors. Material from the first 6 passages was used for the present work.

LNSA originated in an (A × A.SW)F_1 male, implanted subcutaneously with a pellet containing 1 mg diethylstilbestrol plus 4 mg cholesterol 5 months earlier (*2*). It was propagated in (A × A.SW)F_1 hybrids through 28 passages, interrupted by one period of frozen storage after the 21st passage (*6*). Data concerning its transplantability to mice of the parental strains and other genotypes and the isolation of isoantigenic variants compatible with one of the parental strains have been given previously (*2*).

Antiserums.—We prepared antiserums by inoculating mice of certain genotypes with pooled suspensions of liver, spleen, and lymph-node cells from the appropriate foreign genotype. Between 15 and 20 subcutaneous and intraperitoneal inoculations were given at weekly intervals. The mice were bled from the retro-orbital sinus 4 to 6 days after the last injection. Serums of several mice were pooled and stored at −20° C. The antiserums were absorbed by incubation of 0.5 ml of a serum, diluted 1:5 in Ringer's solution, with approximately 10^9 packed spleen and lymph-node cells at room temperature for 30 minutes. The tubes were agitated in a vibrator during the absorption procedure. Two sequential absorptions were sufficient to remove all detectable cytotoxic activity against the *H-2* type of the absorbing cells.

Four different antiserums were prepared: A-anti-A.SW, A.SW-anti-A, (A.SW × DBA)F_1-anti-A, and (C3H × C57BL)F_1-anti-A. The *H-2* antigens involved in these reactions, according to Amos (*7*), are shown in table 1. The A.SW-anti-A serums can be expected to contain antibodies directed against the *H-2* isoantigens D, K, and Y. In addition, they may possibly contain antibodies against A, J, M, and N, antigens present in A but not identified in A.SW. Allowance has to be made for other antigens not yet identified. Absorption with C3H (*H-2*^{k}) cells removes activity against K and Y, but leaves anti-D activity. Absorption with DBA (*H-2*^{d}) cells removes anti-D (and, if present, anti-J, anti-M, and anti-N), but leaves anti-K and anti-Y; the latter antibodies can also be expected to be present in the A.SW × DBA-anti-A serum. The C3H × C57BL-anti-A serum would, after absorption with A.SW cells, contain anti-D and, possibly, anti-J and anti-M.

The best known and probably strongest antigens involved in this study

are D and K. They are of primary importance for the homograft reaction against strain A tumors, and can easily be demonstrated serologically (*7*). They can be separated by meiotic crossing over (*8*). According to Gorer, the *H-2*^{a} complex may have arisen by meiotic crossing over of *H-2*^{d} and *H-2*^{k} (*8*). As pointed out in the preceding paper (*1*), it is therefore probable that the *H-2* antigens of strain A, which are shared with DBA/2, belong to one group and those shared with C3H to another. For convenience and simplicity these two groups of antigens will be referred to as D and K, as in the preceding paper (*1*), and the corresponding antiserums as anti-DK, anti-D, and anti-K, respectively.

All serums were tested against normal lymphocytes before and after absorption. To obtain comparable results, the same pools of frozen antiserums were used for all experiments.

Serologic tests.—The cytotoxic test of Gorer and O'Gorman was used (*4*). For suspension of cells, the tumors were pressed through a 60-mesh stainless-steel screen into Ringer's solution, containing 100 IU of penicillin and 100 γ streptomycin per ml. Suspensions of normal lymphoid cells were obtained from lymph nodes in a similar way. All suspensions were washed twice with Ringer's solution and resuspended to a final concentration of about 10^7 cells per ml. Equal volumes (0.05 ml) of cell suspension, serially diluted isoimmune serum (1:5 to 1:320), and guinea-pig complement were mixed and incubated at 37° C for 90 minutes. The percentage of eosin-unstained cells was determined according to Schrek (*9*) before and after incubation. The cell suspensions were diluted 1:10 with Tyrode's solution containing 0.05 percent eosin, and at least 300 cells were counted rapidly in a hemocytometer and classified as stained or unstained. For each antiserum dilution, a cytotoxic index was computed, defined as the difference between the proportion of unstained cells in the control and the test suspension, divided by the former figure. The titer shown corresponds to the highest dilution of antiserum that still gave a cytotoxic index of at least 0.15.

RESULTS

Cytotoxic Tests on Normal Lymph-Node Cells

All serums used against tumor cells were tested against normal lymph-node cells first. The results are shown in table 1. All serums tested in a 1:5 dilution were toxic for cells containing the target antigens, the cytotoxic indexes varying between 0.60 and 1.0. When cells of different genotypes were tested against the same antiserum, titers differed between 1:40 and 1:160. Homozygous cells were more sensitive and gave a higher titer than corresponding heterozygous F_1 hybrid cells, in agreement with previous findings (*2*). In addition, C3H cells appeared to be somewhat more sensitive than DBA cells when tested against the same A.SW-anti-A serum.

TABLE 1.—Summary of serologic tests performed with normal lymph-node cells

Lymph-node cells		Unstained cells in control (%)	A.SW-anti-A				A.SW × DBA-anti-A				A.SW-anti-A, absorbed with DBA cells			
				1:5				1:5				1:5		
Origin	Known *H-2* antigens*		Reaction possible against*	Unstained cells (%)	CI†	Titer	Reaction possible against*	Unstained cells (%)	CI†	Titer	Reaction possible against*	Unstained cells (%)	CI†	Titer
A	ACDEFHJK-MNY	49	DKY(AJMN)	2	0.96	1:160	KY(A)	3	0.93	1:80	KY(A)	1	0.98	1:80
		58		11	0.81	1:160								
(A × A.SW)F1	ACDEFHJK-MNY/CEFGHS	66	DKY(AJMN)	8	0.88	1:80								
A.SW	CEFGHS	62												
DBA/2	CDE^{d}FHJMN	50	D(JMN)	0	1.0	1:40						54	0	<1:5
		62		5	0.92	1:40		58	0	<1:5		49	0.21	1:5
		72										75	0	<1:5
C3H	ACEHKY	57	KY(A)	7	0.88	1:80	KY(A)				KY(A)	5	0.91	1:80
		52		1	0.98	1:80						21	0.60	1:40
		80										18	0.77	

Lymph-node cells			A.SW-anti-A, absorbed with C3H cells				C3H × C57BL-anti-A, absorbed with A.SW cells				A-anti-A.SW			
				1:5				1:5				1:5		
Origin	Known *H-2* antigens*	Unstained cells in control (%)	Reaction possible against*	Unstained cells (%)	CI†	Titer	Reaction possible against*	Unstained cells (%)	CI†	Titer	Reaction possible against*	Unstained cells (%)	CI†	Titer
A	ACDEFHJK-MNY	49 58	D(JMN)	6	0.88	1:40	D(JM)	0	1.0	1:80				
(A × A.SW) F_1	ACDEFHJK-MNY / CEFGHS	66									GS	0	1.0	1:80
A.SW	CEFGHS	62						60	0	<1:5	GS	0	1.0	1:160
DBA/2	CDE^dFHJMN	50 62 72	D(JMN)	5 9 17	0.90 0.85 0.76	1:40 1:40	D(JM)	2	0.98	1:80				
C3H	ACEHKY	57 52 80		52 49 81	0 0 0	<1:5 <1:5 <1:5								

*The *H-2* isoantigens are given according to Amos (7).
†CI = cytotoxic index.

Two absorptions of the A.SW-anti-A serum with DBA cells completely removed all detectable cytotoxic activity against DBA; C3H cells were still sensitive to a titer of 1:40 to 1:80. The result was analogous when absorptions with C3H were performed. There were no major differences between the effect on DBA or A cells of C3H × C57BL-anti-A serum, absorbed with A.SW cells, or A.SW-anti-A serum, absorbed with C3H cells, if comparison is made between the cytotoxic indexes at the dilution 1:5, though the titers were higher with the former serum (1:80 as compared to 1:40). They were later used interchangeably as anti-D serums to test tumor cells, though usually the tumor cells were tested simultaneously against both serums. For similar reasons, A.SW × DBA-anti-A and A.SW-anti-A serums, absorbed with DBA cells, were used interchangeably as anti-K serums.

Cytotoxic Test on Lymphomas

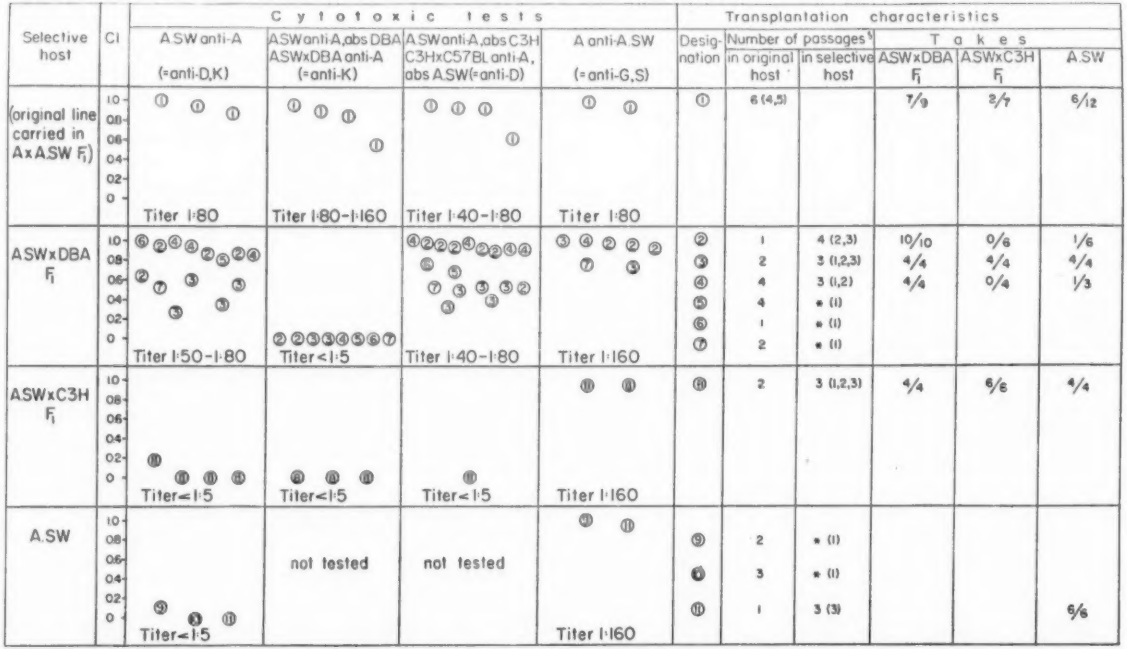
Various samples of LNSF and LNSA, including unselected lines, carried in the original (A × A.SW)F_1 hybrid type and variant sublines isolated in selective hosts, *i.e.*, (A.SW × DBA)F_1, (A.SW × C3H)F_1, and A.SW mice, were analyzed. The *H-2* antigenic composition of the original and selective hosts appears in table 1 of the preceding paper (*1*) and will not be repeated.

The transplantation behavior of LNSA has been described previously (*2*). It has a strong tendency for "nonspecific growth," violating *H-2* barriers, and growing progressively in the parental strains and foreign genotypes as well. This nonspecific growth could be largely prevented by the use of hosts preimmunized with tissue containing *H-2*^{a}, derived from strain A. In the present study, all nonisologous recipients of LNSA were preimmunized by a subcutaneous inoculation, about 2 months earlier, of an incompatible A or (A × A.SW)F_1 tumor that had completely regressed before LNSA was inoculated.

LNSF showed a more specific transplantation behavior and untreated hosts were used as recipients throughout.

Text-figure 1 summarizes the transplantation data and the cytotoxic tests with LNSF. LNSF appears to possess both D and K, since it was killed both by anti-D and anti-K serums. Cytotoxic indexes at serum dilution 1:5 were between 0.57 and 0.96. Titers ranged from 1:40 to 1:160.

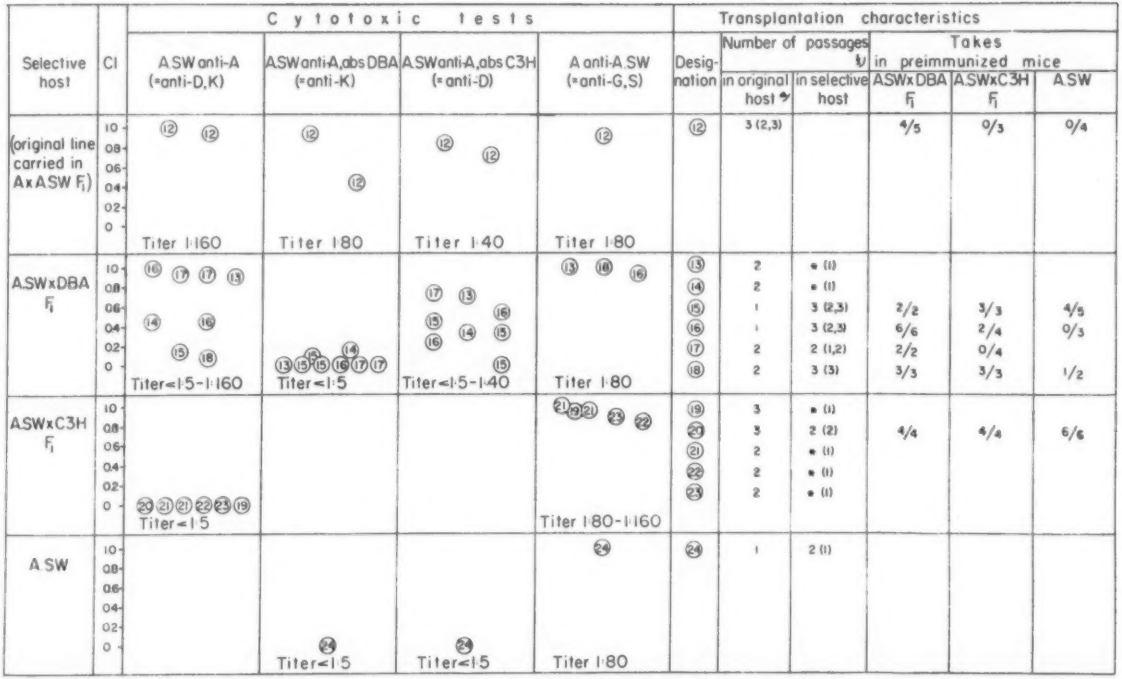
Six LNSF variants isolated in the selective (A.SW × DBA)F_1 mice, three of which were carried by serial passage in the same host, were completely resistant to anti-K serum. They varied in their susceptibility to anti-D serum; all were susceptible but to different degrees. Sensitive cells, like the original unselected line, were killed at a titer of 1:40 to 1:80. It is of interest to compare the transplantation behavior of the three sublines tested (②, ③, and ④) with their sensitivity to anti-D serums. Sublines ② and ④ were as sensitive to anti-D serum as the unselected line. They grew in all (A.SW × DBA)F_1 hybrids, but in no (A.SW ×



TEXT-FIGURE 1.—Summary of serologic and transplantation tests performed with lymphoma LNSF. The cytotoxic indexes (CI) as obtained with 1:5 antiserum dilution are plotted at *left*. Available transplantation results are at *right*. Figures denote the number of mice killed by progressively growing tumors, as related to the total number inoculated. Numbers in *circles* denote independent sublines or independently isolated takes in selective hosts, inoculated with the unselected line.

* Take of original line in selective host.

§ Number of serial transfers preceding cytotoxic test is indicated in parentheses.



Selective host	Designation	in original host §	in selective host	A SW x DBA F1	A SW x C3H F1	A SW
(original line carried in A x A SW F1)	⑫	3 (2,3)		4/5	0/3	0/4
A SW x DBA F1	⑬	2	* (1)			
	⑭	2	* (1)			
	⑮	1	3 (2,3)	2/2	3/3	4/5
	⑯	1	3 (2,3)	6/6	2/4	0/3
	⑰	2	2 (1,2)	2/2	0/4	
	⑱	2	3 (3)	3/3	3/3	1/2
A SW x C3H F1	⑲	3	* (1)			
	⑳	3	2 (2)	4/4	4/4	6/6
	㉑	2	* (1)			
	㉒	2	* (1)			
	㉓	2	* (1)			
A SW	㉔	1	2 (1)			

TEXT-FIGURE 2.—Summary of serologic and transplantation tests performed with lymphoma LNSA. The cytotoxic indexes (CI) as obtained with 1:5 antiserum dilution are plotted at *left*. Available transplantation results are at *right*. Figures denote number of mice killed by progressively growing tumors, as related to total number inoculated. Numbers in *circles* denote independent sublines or independently isolated takes in selective hosts, inoculated with the unselected line.

§ Number of serial transfers preceding cytotoxic test is indicated in parentheses.

▲ LNSA was propagated during 25 passages in (A × A.SW)F1 mice, before these tests were carried out.

* Take of original line in selective host.

C3H)F_1 mice and only in a fraction of A.SW. On the other hand, subline ③ was less sensitive to anti-D serum than the others. It was killed to a cytotoxic index of 0.38 to 0.55, though the titer was 1:40 to 1:80. This line grew equally well in A.SW × DBA, A.SW × C3H, and A.SW mice. It can be surmised that it represents a mixture of cells that have lost both D and K and become compatible with all three types of hosts, and cells that have only lost K and would be compatible with (A.SW × DBA)F_1 alone, if present in a pure population. Variants ② and ④ can then be regarded as representative of such pure populations, with only K missing.

Subline ⑧ isolated and carried in (A.SW × C3H)F_1 mice, was resistant to A.SW-anti-A serum as well as to C3H × C57/BL-anti-A and A.SW × DBA-anti-A serums. This indicates that it had lost both the antigens D and K, in agreement with its transplantation behavior: It grew in all (A.SW × C3H)F_1 and A.SW mice inoculated. Three tumors isolated in A.SW mice were all resistant toward A.SW-anti-A serum, similar to the subline isolated in A.SW × C3H.

All different LNSF lines studied serologically were killed by an A-anti-A.SW serum in all tests performed to cytotoxic indexes of 0.73 to 1.0. In agreement with previous findings, the variants were sensitive to a higher titer than the original, unselected LNSF: 1:160 as compared to 1:80.

The serologic tests performed with LNSA, summarized in text-figure 2, gave results similar to those obtained with LNSF and will therefore be commented on more shortly. None of six (A.SW × DBA)F_1 variants had any detectable K antigens, but all of them except ⑱ were killed by anti-D serums. In two cases (⑮ and ⑱) resistance toward anti-D serums, in the majority, respectively, all cells could be correlated with ability for growth in A.SW and (A.SW × C3H)F_1 mice, preimmunized against A, while in another case (⑰) a subline which did not grow in preimmunized A.SW × C3H or A.SW mice contained cells killed to a cytotoxic index of 0.75 by anti-D serum. None of five (A.SW × C3H)F_1 variants, four of which were primary takes after inoculation of unselected LNSA, had any detectable D or K antigens.

DISCUSSION

The possible mechanisms of isoantigenic variant formation in F_1 hybrid tumors have been discussed in detail in previous publications (*2, 3, 10–13*) and in the preceding paper by Klein (*1*). This discussion can be restricted to comments on the results obtained in the present study.

The cytotoxic test of Gorer and O'Gorman (*4*) was used to analyze some *H-2*-determined isoantigens of two lymphomas, of (A × A.SW)F_1 origin, and their variants isolated in (A.SW × DBA)F_1, (A.SW × C3H)F_1, and A.SW mice. The results of repeated experiments on the same sublines were consistent.

§ Number of serial transfers preceding cytotoxic test is indicated in parentheses.
◀ LNSA was propagated during 25 passages in (A × A.SW)F_1 mice, before these tests were carried out.
* Take of original line in selective host.

The majority of unselected cells of LNSF and LNSA, taken from (A × A.SW)F_1 mice, were killed by both anti-D and anti-K serums. Twelve independent takes in (A.SW × DBA)F_1 mice were studied, 7 of which were carried further in (A.SW × DBA)F_1 mice through 1 to 2 passages and also tested for growth in A.SW × DBA, A.SW × C3H, and A.SW mice. They all consisted of cells completely resistant against anti-K serums, which indicated the loss of K. On the other hand, anti-D serums killed a varying fraction of cells in 11 of the 12 variants. Some of them were as sensitive as unselected LNSF; others showed a decreased sensitivity. While the former were specifically compatible with A.SW × DBA and did not grow in A.SW × C3H, the latter type consisted of a mixture of two cell types having lost K alone and both D and K, respectively.

Six independent takes in (A.SW × C3H)F_1 mice, studied either directly or after 1 to 2 transfers in A.SW × C3H, consisted of cells that were resistant against both anti-D and anti-K serums. They were in this respect similar to variants isolated in A.SW, in agreement with the few transplantation tests performed, and had probably lost both D and K antigens.

In preceding papers it has been pointed out that the formation of isoantigenic variants from F_1 hybrid tumors is probably the result of immunoselection of variant cell clones, either pre-existing or arising during the period of background growth (*2*, *11*, *12*). Possible epigenetic changes, induced by anti-*H-2* antibodies, could not be entirely excluded, however (*2*). The fact that the variants isolated in (A.SW × C3H)F_1 mice had lost both D and K, though the selective host could not form anti-K antibodies, speaks against this. In A.SW × DBA hosts, which cannot form anti-D, variants lacking D can sometimes be isolated as shown in the present as well as in the preceding paper (*1*). In such mice variants also appear, which presumably consist of a mixture of cells, having or lacking D. Such findings argue strongly against antibody-induced epigenetic changes and favor the immunoselection hypothesis.

The results of this study agree with the findings of Klein, reported in the preceding paper (*1*). In the systems used, antigen K can be lost alone or with D, but antigen D cannot be lost without K. This strengthens the hypothesis that isoantigenic variant formation from F_1 hybrid tumors is due to somatic crossing over (*1–3*). To exclude other possible but less likely explanations of the results obtained, *e.g.*, chromosomal deletion. studies with more markers on the same chromosome are needed.

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Specific Localization *In Vivo* of Antihepatoma Antibodies in Autochthonous Rat Hepatomas [1, 2]

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SUMMARY

Radioiodinated antihepatoma antibodies, after purification *in vivo* from induced hepatomas in rats, localized in the total hepatic region (liver with hepatoma) of hepatoma-bearing rats to an extent as high as 60 percent of the injected dose. The localized antibodies were shared almost equally by hepatoma and non-neoplastic portions. When the purified antibodies were passively transferred through normal rats, cross-localizing antibodies were absorbed from the circulation. Antibodies that remained in the serum, when assayed in hepatoma-bearing rats, preferentially localized in discrete hepatomas and localized to as high as 40 percent of the injected dose in the total hepatic region. When assayed in normal rats, the same preparation localized at the 4 percent level in liver. Antihepatoma antibodies, when purified *in vivo* from normal liver of normal rats and assayed in hepatoma-bearing rats, preferentially localized in the non-neoplastic portions of the hepatic region.—J. Nat. Cancer Inst. 27: 1107-1114, 1961.

INDUCED RAT HEPATOMAS trap circulating antihepatic tissue antibodies nearly as well as the non-neoplastic portions of liver (*1*, *2*). Unlike transplanted hepatomas, these autochthonous liver tumors are supplied with a well-developed undamaged vasculature; consequently, they can be used as a suitable test system for the study of localizing antitumor antibodies (*3*).

Radioiodinated antibodies which localize *in vivo* in the hepatomas can be recovered by the following steps: (*a*) perfusion and excision of discrete tumor portions, (*b*) preparation of saline-insoluble (*p*H 8) tissue sediments, (*c*) elution of the radioactive protein by heating at 60 to 65° C for 20 to 30 minutes, and (*d*) centrifugation at room temperature or lower to obtain a clear supernatant fluid that contains the eluted portion. We term this procedure "purification *in vivo*." It is similar to the

[1] Received or publication, May 26, 1961.

[2] This work was supported in part by research grant C-4633 from the National Cancer Institute, National Institutes of Health, Public Health Service, and by contract AT(30-1)-2651 with the Atomic Energy Commission.

[3] The authors wish to acknowledge the assistance of Mrs. Hilda Kantor, Mr. Andrew Z. Barancewicz, and Mr. Wassyl Potischko.

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procedure developed previously to purify antibodies capable of localizing in normal tissues (*4*). Antihepatoma antibodies, which are purified *in vivo* and tested for their localizing properties in a second group of hepatoma-bearing rats, reveal an increased propensity for hepatic tissue in general but only an insignificant increase in specificity for hepatoma *per se*. The large amount of cross-reaction that these purified antihepatoma antibodies have for non-neoplastic liver portions emphasizes the antigenic similarities between hepatomas and the remaining hepatic tissue and underscores the difficulty of obtaining antibodies that do localize specifically in hepatomas.

Added to this major problem of cross-localization of antihepatoma antibodies in liver are two secondary problems that make the quantitative determination of hepatoma localization more complicated than, for example, kidney localization. First, it is rare to obtain non-neoplastic liver portions free from micronodules of hepatoma. These bits of tumor populate the otherwise non-neoplastic liver to an extremely variable extent, compete with the discrete portions of hepatoma for antibody, and make estimates of the total hepatoma-localizing antibody, as distinct from the total non-neoplastic liver-localizing antibody, a very special problem indeed. The second problem is created by the variability from rat to rat in the mass not only of discrete hepatomas but also of non-neoplastic liver portions which contain bile-duct proliferations and cirrhotic lesions.

The first part of the experimental work contains a summary of the type of data which quantitatively expresses hepatoma localization of antibody. By the use of the mathematical expression, as shown in the second part of the experimental account, we offer proof of the existence of some antibodies that will localize preferentially in hepatoma, and other antibodies that will localize preferentially in non-neoplastic liver.

MATERIALS AND METHODS

Hepatomas were induced in Sprague-Dawley (Charles River CD) male rats by *N*-2-fluorenylacetamide. Antiserums were prepared in groups of 4 rabbits. The serums from each group were pooled. Antiserum globulins were obtained by salting out with sodium sulfate. Radioiodination was limited to 100 μc per mg globulin and to 1 atom of carrier iodine per protein molecule. For purification *in vivo*, the time varied from 1 to 5 days between injection of radiolabeled whole globulin and recovery of localized radioantibody. For assay of all purified antibody preparations in second groups of rats, the time between injection and killing was kept at 1 day. All tissues, whether for use as antigens, as media for recovery of antibody after purification *in vivo*, or for assay, were perfused exhaustively, before excision, with 0.90 percent NaCl with the aorta as point of entry. Details of all these procedures have appeared (*1*).

QUANTITATIVE ASPECTS OF ANTIBODY LOCALIZATION IN HEPATOMAS

Four pools of antihepatoma antiserum globulin were the source of antibody. The antiserums of pool 1 were prepared against the dense sediments[4] of discrete hepatomas; of pool 2, against whole homogenates of whole hepatic tissue from hepatoma-bearing rats; of pool 3, against the dense sediments of livers populated in their entirety with hepatomas; and of pool 4, against the light sediments[4] of discrete hepatomas. Each of the 4 pools of antiserum globulin was tested 3 times in groups of 4 rats. Forty-eight hepatoma-bearing rats were assayed in all.

For each test an antiserum globulin was purified *in vivo* from the dense sediment of discrete hepatomas, and the recovered portion was injected intravenously into 4 hepatoma-bearing assay rats. In terms of the injected radioactive dose, the purified antibodies in the combined 12 tests localized in the total hepatic zone (neoplastic plus non-neoplastic) to an extent as high as 61 percent, and averaged 31 percent. The extent of localization depended mainly on the time period between injection and recovery of globulin in the purification step. As the period increased, the nonspecific radioactive globulin within the hepatomas was reduced; thus, the localized antibody that remained in the tissue (and subsequently recovered) was enriched. Discrete hepatomas ranged in weight from 3 to 19 g; total hepatic tissue, from 22 to 48 g. The results of 3 of the 12 tests, those of antihepatoma antiserum pool 1, are shown in table 1. The results of the other 9 tests are similar. The problems mentioned in the introduction are all seen here: the variability in size of discrete hepatomas and of the total hepatic tissue, the variability in antibody localization in the hepatomas, and the extensive cross-reaction that is expressed by the difference between discrete neoplastic and total hepatic localization. Within a group of 4 rats receiving a single preparation total hepatic localization appears more or less constant, but from one preparation to another even this parameter is subject to variation.

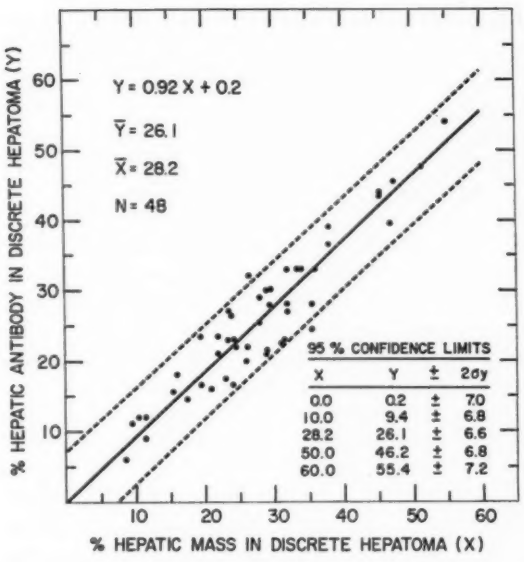
The one consistent feature, which the data for the 3 tests of antihepatoma antiserum pool 1 and the data for the 9 tests of the other 3 pools reveal, is the functional relationship between the mass of a discrete hepatoma and the amount of antibody contained in it when these values are expressed in terms of the percentage of the total hepatic mass and the percentage of the total antibody localized in the hepatic zone. The last two columns of table 1 illustrate this correlation for the 3 tests of antihepatoma antiserum pool 1 in 12 assay rats, and the plot of data in text-figure 1 graphically demonstrates the correlation for the 12 tests of the 4 antibody pools in the 48 assay rats. The regression line, calculated by the method of least squares, and the 95 percent hyperbolic confidence limits about the

[4] Preparation of dense and light sediments have been described (*1*). Both are well-washed, low-speed ($100 \times g$) insoluble debris of homogenates of perfused tissues. Dense sediments are mainly connective tissue and blood-vessel stroma, and are light-colored and fibrous. Light sediments are mainly parenchymal-cell debris, are liver-colored, and become gelatinous upon heating.

TABLE 1.—Variability in mass and antibody localization* in discrete hepatomas of individual rats as compared to the total hepatic region

Test	Mass hepatic tissue (g)		Percentage injected dose in hepatic tissue*		Percent hepatic mass in discrete hepatoma	Percent hepatic antibody in discrete hepatoma
	Discrete hepatomas	Total hepatic region	Discrete hepatomas	Total hepatic region		
1	8. 35	34. 87	9. 98	60. 81	23. 9	16. 4
	5. 52	35. 32	8. 20	52. 91	15. 6	15. 5
	13. 50	39. 47	17. 03	51. 83	34. 2	32. 9
	6. 61	30. 22	10. 67	50. 40	21. 9	21. 2
2	9. 65	30. 12	11. 43	41. 08	32. 0	27. 8
	19. 30	40. 90	15. 90	40. 20	47. 2	39. 6
	11. 82	42. 82	8. 73	39. 56	27. 6	22. 1
	13. 58	38. 40	13. 54	39. 19	35. 3	34. 5
3	19. 20	40. 54	9. 58	21. 07	47. 4	45. 4
	8. 80	30. 00	6. 26	20. 97	29. 3	29. 9
	9. 54	28. 41	6. 81	20. 75	33. 6	32. 8
	9. 79	37. 32	4. 13	20. 42	26. 2	20. 2

*The antibodies used in this table are from pool 1; data for the other 3 pools were similar. For each test, radioiodinated antihepatoma antiserum globulin was purified *in vivo* from hepatomas of donor rats, and the recovered antibody was injected into 4 hepatoma-bearing rats for assay (*see* text). The antibody assayed in the first test had been in the donor rats for 5 days; in the second test, 2 days; in the third test, 1 day. The longer the localized antibody resided in the donor rats, the richer it became, since nonspecific labeled globulin steadily decreased in amount.



TEXT-FIGURE 1.—Correlation between hepatoma mass (as % of the total hepatic region) and amount of antibody localized in the hepatoma (as % of the antibody in the total hepatic region). The regression line was calculated by the method of least squares; the 95 percent hyperbolic confidence limits were calculated for Y, given any X.

ordinate define the scope of usefulness of this quantitative mathematical expression of hepatoma localization.

Purified antibody preparations from any of the 4 pools of antiserum globulin that, after further treatment and analysis, assay consistently above the upper 95 percent confidence limit may be said to contain antibodies with an increased propensity for hepatoma. Those that assay below the lower 95 percent confidence limit may be said to contain antibodies with an increased propensity for non-neoplastic liver. The method is, of course, limited to the assay of antibody in hepatic tissues in which the distributions of neoplastic and non-neoplastic tissues are nonuniform and different from each other. Fortunately, livers homogeneously populated with hepatomas are not too common and livers containing discrete hepatomas are relatively frequent.

SPECIFIC LOCALIZATION OF ANTIBODIES IN HEPATOMAS

Passive transfer experiments.—Antihepatoma antiserum globulin pool 1 was the source of antibody. In one experiment the globulin was purified *in vivo* from discrete hepatomas of 3 rats, and the recovered antibody was injected into 3 normal rats. After 1 day the serum from these normal

rats was injected into 4 hepatoma-bearing rats and 4 normal rats for assay. In two other experiments the antiserum globulin was first passed through normal rats and then purified *in vivo* from discrete hepatomas. In the first of these two experiments, the period between injection of the passively transferred antibody into hepatoma rats and recovery from hepatoma was 1 day; in the second, 5 days. These preparations were also assayed in groups of 4 rats, both hepatoma-bearing and normal. In a fourth experiment the antiserum globulin was passed through three sets of normal animals with 1 hour's residence in each set and was then purified *in vivo* and recovered from hepatomas after a residence of 5 days in the hepatoma-bearing rats. The recovered antibody was assayed in 3 hepatoma-bearing rats and 2 normal rats. Less than 1 percent of the original antibody that localized in the hepatic region was recovered in each of the four experiments; however, the amount of radioactivity in the recovered material was more than adequate for the assays.

The recovered antibody in each case was tested in normal rats to determine the extent of residual normal-liver localizing antibody still present in passively transferred material. For the preparation described in table 2 about 10 times as much antibody localized in the hepatic region of tumor-bearing rats (38%) as in normal liver of normal rats (4%). The same preparation before passive transfer localized 52 percent in the hepatic region of hepatoma-bearing rats, and 51 percent in normal liver of normal rats. In none of the passive transfer experiments was antibody, with a potential for localizing in normal liver, completely cleared from the circulation of the donor rats. Anywhere from 1 to 7 percent of the circulating radioactivity of the donor rats would localize in normal liver of recipient rats over and above a nonspecific background localization of about 1 percent.

TABLE 2.—Localization of specifically purified antihepatoma antibody in the hepatic region of normal and hepatoma-bearing rats*

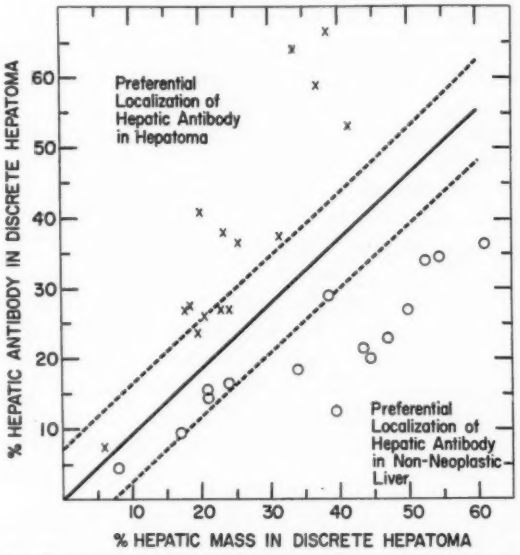
Normal liver of normal rats		Hepatic region of hepatoma-bearing rats	
Percent injected radioactive dose	Weight (g)	Percent injected radioactive dose	Weight (g)
4.3	18.3	38.5	31.3
4.2	15.8	40.1	34.3
5.2	16.3	38.4	33.6
3.9	17.5	37.2	30.9

*Kidney, spleen, lung, thyroid, and blood values were at normal globulin control levels.

It was not enough, of course, to have shown a low localization of specifically purified antihepatoma antibody in normal liver of normal rats and a high localization in the cancer-riddled hepatic region of hepatoma-bearing rats as proof of specific hepatoma localization. Higher hepatic localization in the cancer-bearing host could conceivably have been asso-

ciated with increased bile-duct proliferation, the onset of cirrhosis, or some other non-neoplastic pathology that often accompanies hepatomas. One needed also to show preferential localization of antibody in discrete hepatoma nodules over that in the remaining hepatic region.

As shown in text-figure 2, preferential localization of hepatic antibody in discrete hepatoma was obtained in the 4 experiments in 10 of the 15 rats (an "X" for each individual rat). The scatter of points reflected the



TEXT-FIGURE 2.—Preferential localization of antibodies in hepatomas (X) after passive transfer through normal rats, and in non-neoplastic liver (O) after purification from normal liver. The lines are taken from text-figure 1.

variation in the number of micronodules of hepatoma outside the discrete hepatomas and the variation in the size of the discrete portions. The samples of hepatoma in 3 of the rats in which significant preferential localization was not obtained were too small to bring out differences. Histological examination of the tissues of the remaining 2 showed little difference between the discrete samples and the remaining hepatic tissue that was heavily populated with tumor. Because the micronodules of tumor also localized antibody and consequently decreased the localization in the discrete nodules, the values reported for tumor localization in the discrete nodules were necessarily minimal.

SPECIFIC LOCALIZATION OF ANTIBODIES IN NON-NEOPLASTIC PORTIONS OF HEPATIC TISSUE

Purification in vivo *from normal liver.*—Antihepatoma antiserum globulin pool 1 was the source of antibody in 3 experiments, pool 2 in

a fourth. In all experiments the globulins were purified *in vivo* from normal rat liver of normal rats. The recovered antibody preparations, comprising about 10 percent of the original antibody that localized in the hepatic region, were assayed in a total of 15 hepatoma-bearing rats. As shown in text-figure 2, preferential localization of hepatic antibody in non-neoplastic liver was obtained in 9 of the rats (an "O" for each rat). In the other 6, the samples of hepatoma were either too small or not discrete enough to bring out sufficient differences from the regression line.

DISCUSSION

In these experiments we have been able to demonstrate the antigenic similarity between hepatomas and other hepatic tissue elements. We have also been able to demonstrate some differences that have made it possible for us to obtain specific localization of antibodies in hepatomas, on the one hand, and in non-neoplastic liver, on the other. Whether the antigens responsible for the two types of localization specificity are also specific for the tissues involved is not known. Localization depends on accessibility of the antigens to the circulation; localization specificity, therefore, is an index of the specific exposure to the circulation of a given set of antigens in the tissue in question. Antibodies can, at best, differentiate between sets of antigens that have been made available. Once the antigens have become known they can be sought in other areas that may not be so accessible. If their absence in all but the given tissue is proved, then and only then can true specificity be claimed.

The demonstration of the specific localization of antibodies in autochthonous rat tumors is encouraging in itself. To obtain antibodies specific enough to act as carriers of diagnostic and therapeutic amounts of radioactivity to at least some types of autochthonous neoplasms now comes within the realm of possibility.

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Inactivation by Electron Radiation of the Capacity of Goldenrod Polysaccharide to Induce Vacuolization in Ascites Tumor Cells [1]

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SUMMARY

A polysaccharide from goldenrod was exposed to various doses of 7 kv electrons from a tungsten filament. The attenuation of the capacity of this polymer to induce cytoplasmic vacuolization and enlargement in Sarcoma 37 ascites tumor cells was studied. The logarithm of survival for this property was found to be a nearly linear function of electron dose, within the limits of experimental error. No negative curvature near zero dose was observed. Dose-survival curves for the inactivation of the enzymic functions of ribonuclease (mol. wt. 13,700) and invertase (mol. wt. 120,000) were obtained in the same manner. A comparison with these curves leads to the conclusion that the molecular weight of the polysaccharide moiety responsible for cell vacuolization is in the order of 8,000.—J. Nat. Cancer Inst. 27: 1115–1121, 1961.

WHEN SPECIFIC biologic functions of macromolecules are studied, the question arises as to how much of the total molecule is vital for the performance of the function. This is tantamount to asking how large a molecular weight is associated with a particular moiety. The site of enzymic activity for some globular proteins has been shown to include a small part of the total structure. Nevertheless, some studies show that the approximate volume of the entire molecule is sensitive to inactivation, for it is the three dimensional configuration which positions the site so as to obtain maximum catalytic activity. Agents exist which can disrupt the globular configuration in such a way that the force field around the active site is irreversibly distorted. For example, ionizing radiation, which destroys the integrity of the comparatively frail folded-chain structure of the protein, destroys its ability to function as an enzyme (*1*). Similar studies with deoxyribonucleic acid (DNA) show that some biologic functions are associated with rather large molecular configurations (*2*).

Ionizing radiation studies can be made when there is a property which can be attenuated. As a rule, the amount of radiant energy required to decrease the assayed property by a given percent is inversely related to the

[1] Received for publication June 9, 1961.

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size of the moiety responsible for the property (*1*). The great advantage in the study of the enzymes lies in the fact that the property can be a biologic function, enzymic activity.

This report describes the results of the application of this technique to attenuate a property of a polysaccharide which is also a biologic function. The effect produced by the dry *in vacuo* electron irradiation on the ability of a higher plant polysaccharide to induce cytoplasmic vacuolization in mouse ascites tumor cells was studied. Our intention is to show that within the framework of experimental evidence, a standard may be established for obtaining a functional molecular weight for any active moiety. Such a standard should be of value when a molecule has two or more biologic properties.

MATERIALS AND METHODS

The plant polymer was exposed *in vacuo* to 7 kv electrons which were emitted by a hot tungsten filament and accelerated by a grid arrangement. This device is similar to that previously described by Preiss (*3*), the only difference being that the present instrument has fixed electron emitters. It is the same instrument employed in recent irradiated protein, polynucleotide interaction studies (*4*). The specimens to be irradiated were placed on a target plate in such a manner that they could be indexed into the electron beam from outside the evacuated bombardment chamber. Exposures ranged from 9×10^{13} electrons per cm^2 to 50×10^{13} electrons per cm^2. Usually 5 μa were applied uniformly over an area of 5 cm^2, though current densities could be set at any desired value.

The plant polysaccharide was obtained from the goldenrod plant by initial treatment for several hours with a mixture of 5 percent phenol and 1.65 percent trichloroacetic acid, followed by prolonged dialysis, and freeze-drying the soluble dialysate (*5*).

Twenty μg of goldenrod polymer, dissolved in 0.02 cc of distilled water, was placed on ½-inch stainless-steel discs so that the material nearly covered the entire surfaces. The discs were then placed in a desiccator until completely dry. Since the material was of density greater than one, these layers were less than 0.2 μ in thickness, if uniform. Inasmuch as all dose-activity curves obtained showed no positive curvature at high doses, it is inferred that the 7 kv electrons passed readily through such layers.

For each experiment, 54 discs were prepared as described. Twenty-seven discs were exposed to various doses of electrons. Twenty-seven similar discs, which served as nonirradiated controls, were placed in the evacuated chamber outside the beam areas.

When irradiation was completed, the treated and control discs were placed into separate petri dishes containing 2.5 ml pyrogen-free distilled water for 15 minutes to dissolve the polysaccharide. Three CAF_1 mice carrying 3-day-old Sarcoma 37 ascites tumors each received intraperitoneal injections of 180 μg of irradiated polymer; 3 similar mice were each

given 180 μg of untreated polymer, and 3 mice served as untreated controls.

When this untreated goldenrod polymer is injected intraperitoneally into mice carrying Sarcoma 37 ascites, the cells undergo a process of cytoplasmic vacuolization and enlargement which is evident in 8 hours and persists for several days. This phenomenon has been described in detail in a previous communication (*5*). At 24 hours after injection, cells from treated and control mice were aspirated with a glass pipette and mounted between slide and coverslip. They were observed each day for 4 days, with both transmitted light and phase-contrast microscopy. To obtain a quantitative expression of effects on the tumor cells produced by the irradiated samples of polymer, counts of 300 or more vacuolated cells were made daily by two independent observers.

The experiments were repeated five or more times, and the results, which are expressed as the percentage retained potency of the polymer after each electron irradiation dose, are the combined averages of the values for 15 mice.

RESULTS AND DISCUSSION

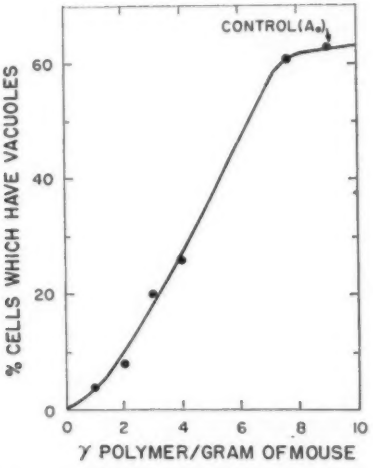
The calibration curve shown in text-figure 1 was obtained for the range of polymer doses injected in the mice. It provided the means by which a count of vacuolated cells was used as an assay for the amount of potent polymer injected into the mice. Such counts were made 24 and 48 hours after injection, and the average of these two figures was taken to obtain the percentage of cells which had vacuoles. These times were arbitrarily selected only because of the vigorous response observed.

The data are presented in table 1. The percentage of vacuolated cells induced by the irradiated polymer is, by means of the calibration curve in text-figure 1, a measure of the amount of potent polymer remaining after irradiation. This amount is given in the next to last column of the table. The percent retained potency is obtained by dividing this figure by the amount of polymer injected into all mice (9γ/g of mouse), and multiplying by 100.

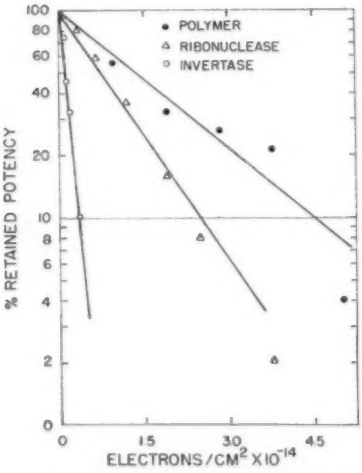
TABLE 1.—Electron inactivation of capacity of goldenrod polysaccharide to induce vacuolization in Sarcoma 37 ascites tumor cells

Radiation dose electrons/cm²	Percent vacuolated cells in untreated controls	Percent vacuolated cells induced by non-irradiated polysaccharide	Percent vacuolated cells induced by irradiated polysaccharide	Retained activity	
				γ Polymer per g of mouse	Percent
0.94×10^{16}	0	64	36	5.1	57
1.88×10^{16}	0.7	60	18	3.0	33
2.81×10^{16}	0.5	64	14	2.5	28
3.75×10^{16}	0.7	66	11	2.1	23
5.0×10^{16}	0.2	60	1.0	0.4	4

In text-figure 2 the electron dose-percent retained potency function for the polymer is compared with those obtained for the enzymatic activities of the two globular proteins, ribonuclease and invertase. The latter molecules were deposited in the same amounts on the irradiation discs and bombarded just as was the polymer. Ribonuclease activity was assayed by the method of Anfinsen *et al.* (*6*) and invertase by the method of Sumner (*7*).



TEXT-FIGURE 1.—The calibration curve for activity versus mass of polymer.



TEXT-FIGURE 2.—The inactivation of the vacuole-inducing capacity of goldenrod polysaccharide compared with those for the enzymic properties of two globular proteins irradiated with 7 kv electrons from the same device.

From the figure it is at once apparent that more ionizations per unit mass of material (on the bombardment discs) are required to attenuate the polysaccharide property by a given amount than are needed to attenuate the specific activities of the two enzymes by the same amount. Furthermore, within the limits of experimental error we can write

$$\log_{10} A/A_0 = -\gamma D$$
$$\text{or}$$
$$A/A_0 = 10^{-\gamma D}, \qquad \text{I}$$

where A is a measured biological activity, A_0 is the control activity, D is the dose of 7 kv electrons expressed as electrons per cm^2, and γ is a constant, different for all three substances. (A calibration curve similar to that in text-fig. 1 has been obtained for the enzymes.)

Now, it is only for convenience that we have measured our doses in such a way that the dimensions of γ turn out to be cm^2 per electron. D is always directly proportional to the energy loss per cm^3 throughout the volume of a thin layer of irradiated material. But the definition of D does not in any way allow us to establish an escape criterion for the molecules of any one substance. This has to be established in either of two ways: 1) the determination of the manner in which the particular radiation loses energy in all materials of interest, or 2) the establishment of an empirical relationship between the arbitrarily defined γ and some property which all molecules of the irradiated substances possess. The former procedure represents an application of what has been called target theory (*1*). Its precision depends on the accuracy with which energy-loss parameters can be specified. Here we use the latter empirical procedure, for we do not claim to know these parameters for the 7 kv electrons. But the loss of precision is offset by a gain of rigor, within the framework of the empirical relationships established.

Let us make a comparison of doses which bring the survival ratios down a given amount, say 1 log. Then

$$-1 = \gamma D_{10\%},$$

and we can use either γ, the line slope, $D_{10\%}$, or the slope reciprocal to compare dose-survival curves. Now the molecular weights of invertase and ribonuclease are known to be 120,000 and 13,800, respectively. Furthermore, the dose-survival curves for these two enzymes (text-fig. 2) suggest that the larger enzyme is more sensitive to inactivation, for $D_{10\%}$ is about 0.32×10^{14} electrons per cm^2 for invertase and about 2.5×10^{14} electrons per cm^2 for ribonuclease.

Recently the 18 S γ_1-globulin human anti-A–anti-B factor was treated with 7 kv electrons from the same device (*8*). A straight target theory analysis yielded a value of 750,000 for the molecular weight, which was previously known to be between 500,000 and 1 million. The 10 percent survival dose turns out to be about 0.5×10^{13} electrons per cm^2. When the protein figures are listed, as in table 2, some interesting features are revealed.

The nearly constant value of the product $MD_{10\%}$ over such a wide

molecular-weight range is the experimental fact on which the escape criterion rests.

Formally,

1) M's are known active masses (for materials of approximately equal densities).
2) D's are proportional to energy loss per unit mass of irradiated material.
3) D's are obtained with the same bombarding particles in all cases.
4) Therefore, since $MD_{10} \approx k$, the damage escape criterion is that on the molecular level, the probability of escape is inversely proportional to the active mass.

TABLE 2.—List of protein figures

Protein	Molecular weight (M)	$D_{10\%}$	$MD_{10\%}$	From
Ribonuclease	13, 800	$2.5 \pm 0.3 \times 10^{14}$	$3.4 \pm 0.4 \times 10^{18}$	Text-fig. 2
Invertase	120, 000	$0.31 \pm 0.04 \times 10^{14}$	$3.7 \pm 0.5 \times 10^{18}$	Text-fig. 2
18 S γ_1-globulin	750, 000	$0.5 \pm 0.1 \times 10^{13}$	$3.8 \pm 0.7 \times 10^{18}$	Ref. (*8*)

Despite the uncertainty at high doses, it is apparent from text-figure 2 that each datum for the goldenrod polymer inactivation lies to the right of the ribonuclease line. Thus, when this product is assumed to obtain M, the molecular weight of the active moiety responsible for cytoplasmic vacuolization is about 8,000. The sedimentation constant of this polysaccharide is 3.3 Svedberg units at 25° C in 1 percent NaCl. This implies that the molecular weight of the polysaccharide is about 10 times (or more) that of the molecular weight of the moiety responsible for the cellular effect. This is similar to the report on DNA in which the basic units responsible for some functions are much smaller than those physically determined as the molecule (*2*). Ambiguity is removed once and for all if we agree to say, "molecular weight of an *active moiety* as determined by, etc." There is no physical reason for assuming that a sedimentation-diffusion determined molecular weight is not the molecular weight of a unit which consists of smaller biologically active units, or in part of inert material as far as a particular function is concerned. What we have obtained may be said to be "a molecular weight by comparison with a known class of molecules, the globular proteins."

Our method will not give information as to the shape or spatial distribution of an active moiety. It could, in fact, consist of two spatially distinct units, the destruction or alteration of either of which inactivates both. This indefiniteness is associated with the fact that we are concerned with an active mass. *We have done no more than assume that ionizing radiation loses energy at about the same rate in two classes of dielectrics which have atoms of about the same atomic weight.* On this energy-density basis, the molecular weight of the active moiety of the polysaccharide is a number near 8,000. Other properties, for example, pyrogenicity, may give entirely different numbers.

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Observations on the Occurrence of Hepatomas in Rainbow Trout [1]

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SUMMARY

Rainbow trout reared in hatcheries throughout the United States suffer from an epidemic of primary liver cancer, which in some instances seems to affect practically 100 percent of the trout population over 3 years of age. The hepatocarcinomas apparently grow rather slowly and are not rapidly killing the trout, since large tumor masses may exist in the liver without seriously interfering with the general well-being of these animals. The carcinomas may directly invade adjacent skeletal muscle and form metastases in the spleen and kidney. Among the various causal agents suspected, virus infection and nutritional factors related to the recent introduction of processed commercial feed have attracted the main attention. The bulk of available evidence at present incriminates some constituent, or constituents, in the feed as a nutritive factor, a chemical additive, or a medicinal agent. The occurrence of this epidemic among edible fish subjected to an artificial nutritive regimen provides a serious warning of the possible future production of a similar cancer epidemic in the human population through an increasing contamination of the human environment with some of the many industry-related carcinogenic chemicals.—J. Nat. Cancer Inst. 27: 1123–1143, 1961.

CANCERS IN wild and domesticated animals possess a distinct ecologic relation to environmental cancers occurring in man because of the identity or similarity of the general types of exogenous carcinogens present in the environment of man and animals [Hueper (*1*, *2*)]. It is obvious that the practical significance of such correlations extends especially to those environmental carcinogenic agents with which members of both groups have constant and lifelong contact, *i.e.*, air, water, and food. During recent decades, a limited amount of scientific curiosity has been applied to a study of the character and scope of cancer hazards associated with the presence of natural and man-made carcinogens in the air and in foodstuffs. Such investigations, however, have remained rather rudimentary concerning the carcinogenic effects on animals that consume or

[1] Received for publication June 13, 1961.

[2] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

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live in fresh and salt water and feed on animal and vegetable matter growing in waters contaminated with carcinogenic agents from natural sources (arsenicals, selenium, and radioactive chemicals), or on carcinogenic agents introduced into them by industrial chemical and radioactive effluents, urban sewage, radioactive fallout, agricultural pesticides, run-off from oiled and asphalt roads, ship fuel and motor exhaust, anti-evaporants, processed and medicated fish feed, molluscacides, algicides, amebicides, ethyl carbamate, and similar economic poisons [Hueper (*3*); Hueper and Ruchhoft (*4*); Hueper and Payne (*5*); Wood (*6*); Hall and Cowen (*7*)].

Carcinogenic polycyclic aromatic hydrocarbons have been isolated from the sludge of waters polluted with industrial wastes and with ship fuel oil [Wedgwood and Cooper (*8*); Borneff and Knerr (*9*)] or from mollusks (barnacles, oysters) living in such waters [Shimkin, Koe, and Zechmeister (*10*); Cahnmann and Kuratsune (*11*)]. Isolated observations have been noted of the occurrence of cancers in animals feeding in water contaminated with radioactive strontium (muskrat) [Krumholz and Rust (*12*)] and with oil refinery effluents (croakers) [Russell and Kotin (*13*)].

Because of the rapidly increasing contamination of public waters with chemicals that might create either directly or indirectly a cancer hazard to both man and animals, a cooperative arrangement was made about 5 years ago by the Environmental Cancer Section, National Cancer Institute, with the Bureau of Sport Fisheries and Wildlife, U.S. Department of the Interior, for obtaining fish with tumors for epidemiologic, etiologic, and pathologic study [Editorial (*14*)]. It was thought that through such systematic and coordinated efforts early warning signs concerning a cancer hazard to man from water contaminants might be discovered which, at the same time, might be useful also for the protection of all forms of wildlife against exogenous, natural and man-made cancer hazards. After the demonstration of a few cutaneous verrucous tumors of apparently viral origin affecting the skin and fins of large-mouth bass and blue gills caught in ponds on the Eastern Shore, the real value of this cooperative venture was shown in the spring of 1960 with the discovery of an epidemic of hepatomas among rainbow trout kept at many federal, State, and private hatcheries in the western and eastern parts of the United States [Thompson (*15*); Rucker, Yasutake, and Wolf (*16*); Nigrelli and Jakowska (*17*); Eyestone (*18*); Wood and Larson (*19*)].

The present report deals with some of the epidemiologic, etiologic, and pathologic observations made in connection with, and as a part of, the comprehensive studies undertaken by various governmental and private investigators for determining the etiology of this epidemic and for providing thereby the basis for the development of rational preventive measures.

The following individuals greatly facilitated these investigations by supplying rainbow, brook, and brown trout, as well as information pertaining to the cultivation and feed of these fish: Mr. Paul E. Thompson, Dr. R. E. Johnson, Dr. S. F. Snieszko, Mr. C. E. Dunbar, Mr. J. D.

Larmyeux, and Dr. R. R. Rucker, Bureau of Sport Fisheries and Wildlife, U.S. Department of the Interior; Mr. G. D. Messerly and Mr. C. Wall, National Aquarium, U.S. Department of the Interior; Mr. Albert Powell and Mr. George Palmer, Maryland Inland Game and Fish Commission. Their help is gratefully acknowledged.

INVESTIGATIVE PROCEDURES

Purpose of the Studies

The discovery of liver tumors among rainbow trout in hatcheries, located in several States of the Rocky Mountain area and in the Appalachian Region and managed by agencies of the Federal Government, various State governments, and private groups, made it necessary to ascertain whether the fish of different hatcheries were affected by the same type of tumor, since they doubtlessly were exposed to different environmental conditions of natural and artificial, especially dietary, origin. Rainbow trout, with and without tumors, obtained from nine hatcheries in Idaho, Washington, Montana, West Virginia, Virginia, and Maryland, as well as from the National Aquarium in Washington, D.C., were therefore histopathologically studied for the establishment of their identity from a morphologic and biologic viewpoint.

It seemed essential also that the distribution of the occurrence of these neoplasms among other members of the Salmonidae family be determined, particularly those other members which were reared under the same environmental and dietary conditions as the rainbow trout and possibly kept over a period of several years in the same troughs, where they had intimate contact with the affected fish, lived in the same water, and received the same feed. For this reason, brook and brown trout up to 5 and 6 years old that were used for brood purposes were obtained from several hatcheries where the cited exposure and environmental conditions prevailed. Such studies were indicated for determining, within limits, the influence of genetic and infectious factors.

All trout studied and obtained from hatcheries had been given various types of commercial, processed feed containing a great variety of food constituents and additives, including medications, and having also appreciable amounts of carbohydrates as basic dietary ingredients. This dietary regimen had been introduced 10 to 20 years ago for economic reasons and particularly as so-called "hot feed" for accelerating the growth rate of the fish. The normal, natural diet of trout and the feed provided previous to this period had been composed mainly of proteinic matter. It was fortunate for the study of a possible dietary influence upon the development of liver tumors in rainbow trout that the rainbow, brown, and brook trout kept at the National Aquarium for 3 to 5 years had been fed almost exclusively a diet of sheep liver.

Biologic and Epidemiologic Data

The observations made are based on an examination of 143 rainbow trout ranging in age from 3 to 72 months, and distributed almost equally between the sexes, 36 brown trout covering an age range from 13 to 35 months, and 73 brook trout between 30 and 43 months old (table 1). One of the brook trout was an albino.

TABLE 1.—Estimated age distribution of trout used in investigation

Months	3–6	7–12	13–24	25–72
Species				
Brook		30		43
Brown			1	35
Rainbow	76		4	63
Total	76	30	5	141

The majority of trout examined, particularly the older fish, were obtained either by special selection among the trout kept in the troughs of the hatcheries because they showed a distinct bulging of the belly or had died as the result of physical trauma sustained during the spawning procedure. The younger fish studied had died, as a rule, from "natural" causes. The trout obtained from the National Aquarium were killed because they were, on account of their dietary regimen, an important material for comparative purposes. The fish reported on, for these reasons, do not represent a random sampling of the rainbow-trout populations investigated. Incidence rates of the hepatomas occurring in these fish populations, therefore, cannot be calculated from the material available, with the exception of that supplied by the National Aquarium, because almost their entire stock of rainbow, brown, and brook trout, which were kept there for several years, was killed for the purpose of this study.

Pathology

Significant variations were not noted in the gross appearance of the livers of the different species of trout as far as their origin from any particular hatchery was concerned. The livers of brook and brown trout were invariably normal, with the exception of the liver of one old brook trout that had a whitish, multicystic node measuring about 1 cm in diameter and involving the upper margin of the organ. On histologic examination this proved to be a cavernomatous angioma.

The livers of the rainbow trout supplied by the National Aquarium also were grossly normal regardless of the age and sex of the fish. On the other hand, distinct variations were observed in the livers of rainbow trout obtained from Federal and State hatcheries of both the eastern and western States. Many livers of this group often displayed severe pathologic lesions which increased in frequency and severity with the relative

age of the fish. The livers of all observed rainbow trout below the age of 12 months were grossly normal. In some of the older fish the livers showed single, few, many or very numerous, pinhead-sized, white nodules which were well circumscribed, as a rule, and were located beneath the capsule, or visible in the cut surface. The majority of these trout, however, displayed rather advanced hepatic changes (figs. 1 to 4). The livers were large-to-huge and distinctly nodular. The nodes protruded often markedly above the surface of the liver and were, in part, well circumscribed, and, in part, ill-defined. Most of the nodes, some measuring up to 10 cm in diameter, were white and medullary in appearance. Others were greenish gray, or grayish brown to brownish yellow, sometimes with reddish mottling. Others displayed a multicystic, cavernous appearance on the cut surface. The cavities of the cysts were filled either with a brownish-yellow, soft matter or with a gelatinous, grayish-green material giving such nodes an appearance similar to that of a colloid goiter. Such advanced lesions were observed in all members of a series of 29 brood trout, which had died after being used for the spawn taking. Since none of the trout surviving such procedures was killed for examination, it has remained uncertain whether the presence of liver tumors in the fish thus affected had produced a lowering of their resistance to traumatic injury. In 2 trout with large liver tumors, the neoplastic mass adhered to the wall of the belly. White medullary strands had infiltrated into the muscle tissue of such areas, as was apparent when they were examined in cross sections. One large, male trout had a small, white nodule in the spleen and a dark red, hemorrhagic gastric mucosa. Bloody fluid was found in the body cavities of 2 trout with liver tumors. The thyroid glands were always free of abnormalities.

The earliest histologically demonstrable lesions in these livers consisted of small focal proliferations of slightly atypical liver cells, somewhat larger and more deeply stained than the surrounding normal liver cells (fig. 5). The livers containing such "adenomatoid" manifestations were, as a rule, without any associated pathologic changes, such as fatty infiltration, periportal fibrosis, or diffuse cirrhosis. However, in one instance, a small focus contained groups or cellular strands of two rows of liver cells embedded in loose connective tissue (fig. 6). Many of the well-advanced and even very large neoplastic masses were composed of similarly shaped, sized, and arranged cells which, in the marginal portions of the nodes, were often not distinctly demarcated from the normal liver tissue. Their malignant, invasive character, however, became definite when in the lumen of large veins (fig. 7) tumor cells were occasionally observed. In other hepatic neoplasms which had a more anaplastic morphology, normal and malignant portions could be distinguished without difficulty, especially where the normal liver structures adjacent to the hepatoma were being compressed by tumor tissue or where a glandular or garland-like type of usually rather large cellular hepatocarcinoma existed (figs. 8 and 9), or where the carcinomatous parts possessed a considerable amount of fibrous stroma (fig. 10). The central portions of some cancers

were highly fibrotic and hyalinized (fig. 11). In a few such cancers an empty mesh of fibrous stroma was present after the neoplastic cellular elements originally filling the meshes had entirely disappeared (fig. 12). In some of the more medullary neoplasms, ill-defined cystic cavities within the masses of cancer cells were filled with light-to-deep-pink-stained hyaline matter (fig. 13). These formations apparently accounted for the colloid goiter-like types of tumor nodes observed grossly. Only one of the carcinomas was composed of hepatocarcinomatous and cholangiocarcinomatous elements (fig. 14). All others were hepatocarcinomas.

An invasion of the skeletal muscle of the belly wall by adherent hepatocarcinomatous masses was demonstrable in one trout, while a small intrasplenic metastasis was evident in another (figs. 15 and 16).

COMMENTS

A comparison of the gross manifestations and their histologic characteristics found in rainbow trout of the eastern hatcheries with those noted in this study, as well as by Nigrelli and Jakowska (*17*), Rucker, Yasutake, and Wolf (*16*), Thompson (*15*), and Wood and Larson (*19*) in rainbow trout of eastern and western hatcheries, establishes the fact that they are identical and that, therefore, the same disease is prevalent throughout the country. The descriptions of hepatomas found during recent years in rainbow trout of hatcheries in northern Italy and in France [Cudkowicz and Scolari (*20*); Levaditi, Besse, Vibert, and Nazimoff (*21*); Besse, Levaditi, Vibert, and Nazimoff (*22*)], moreover, agree well with those recorded for American rainbow trout, and therefore suggest that the same disease and probably identical causal factors operate in the production of this hepatoma epidemic among American and European rainbow trout.

The various observations of early and late lesions indicate that a multifocal process exists which starts with hyperplastic proliferations of liver cells occurring in livers which, as a rule, lack any other pathologic changes, such as fatty infiltration, interstitial inflammatory reactions, periportal fibrosis, and cirrhosis. This process can frequently be demonstrated in rainbow trout less than 1 year old. This finding suggests that the causal agent acts very early in life and possesses a relatively high carcinogenic potency. The subsequent development of these focal lesions leads to the formation of usually ill-defined nodular growths which, because of the absence of cirrhotic and necrotic processes, cannot be considered as being essentially of reparative and regenerative nature, but as being primarily neoplastic. The end-stage of this process is represented by hepatocarcinomas (rarely by cholangiocarcinomas) that have a tendency to undergo extensive necrotizing and hyalinizing changes and which, for this reason, may exhibit extensive fibrotic areas and areas of colloidal proteinic liquefaction. The malignant character of the advanced hepatomas was definitely established by the infiltrative and destructive growth

of these neoplasms, which included a direct invasion of adjacent muscle tissue, and by the formation of metastases subsequent to an invasion by tumor tissue of the lumen of hepatic vessels. There can be no doubt that the evidence we have establishes that rainbow trout suffer from an epidemic of hepatocarcinomas and not merely from "nodular hyperplasias."

The morphologic characteristics of the precancerous and cancerous changes observed, as well as the absence of any neoplastic reactions in the thyroid of these rainbow trout, militate against the concept that they may be of metastatic character and perhaps secondary to neoplastic processes in the thyroid gland. This point deserves attention because of the previously reported occurrence of hyperplastic goiters and thyroid carcinomas in brook trout and rainbow trout in the United States [Marine (*23*); Marine and Lenhart (*24*); Robertson and Chaney (*25*); Gaylord and March (*26*); Nigrelli (*27*)]. It has been suggested that such reactions might be attributable to an iodine deficiency of the water in which these fish were living [Berg, Gordon, and Gorbman (*28*)]. It is not reasonable to assume that such a condition exists in the many hatcheries located in widely separated parts of the country where rainbow trout are affected by liver cancers.

The morphologic uniformity and the general distribution of the hepatic neoplasms support the concept that a common, widely disseminated causal factor is responsible for the occurrence of these reactions. In some hatcheries, certain local environmental conditions, such as a relatively high temperature of the water or possibly the genetic background of the strain of rainbow trout used, may exert a modifying effect on the degree of neoplastic responses noted among the trout and may account for the variations in manifestation, age, and incidence rate of the hepatocarcinomas. It is unlikely, however, that other strictly local factors of known or suspected carcinogenic nature and of natural or artificial origin, such as a natural pollution of the water with arsenic, selenium, and radioactive chemicals, or its artificial contamination with known liver carcinogens, such as chlorinated hydrocarbon pesticides, radioactive effluents, and fallout from nuclear energy operations, are involved in this respect. Since some of the hatcheries receive their water supply from nearby springs, there is little, if any, opportunity for any significant pollution of the water by man-made carcinogens.

Several epidemiologic observations made in this study and in those of other investigators, on the other hand, suggest that genetic factors of species-specific and strain-specific nature may determine to some extent the occurrence and incidence rates of hepatomas among rainbow-trout populations. The findings reported here, and those previously recorded by Snieszko (*29*), indicate that brown and brook trout do not share with rainbow trout the susceptibility to cancer of the liver. Brook and brown trout, when kept in environmental conditions identical with those for rainbow trout, *i.e.*, when they were given the same commercial feed and lived in the same troughs with the rainbow trout, did not develop any

hepatomas or other neoplastic lesions of the liver even when they survived from 3 to 5 years, an age at which rainbow trout exhibited a high hepatoma rate. Nielson (*30*) recently reported that different strains of rainbow trout kept on the same diet exhibited widely differing hepatoma rates. At the present defective state of knowledge, it is questionable, however, whether such observations reflect actual and fundamental strain-related differences in susceptibility to some unknown and unidentified hepatocarcinogen, or whether these findings merely indicate that apparently "refractory" strains develop hepatomas at a later age than the "susceptible" ones. A definite decision can be made only when adequate information becomes available on hepatoma epidemiology among trout of different strains kept under strictly standardized conditions for periods of 3 to 5 years.

The claim concerning a genetic background influencing the occurrence of hepatomas is, moreover, directly related to the allegation that rainbow trout reared in American and foreign hatcheries have been "inbred" for many years and that, for this reason, a special genetic mutation may have been produced. There can be no doubt that the breeding methods used in the propagation of rainbow trout differ basically from those employed in the production of inbred strains of mice, and that it is, therefore, scientifically inaccurate to apply the term "inbred" to the rainbow-trout population of the United States. In fact, it seems uncertain whether even the term "strain" can properly be used in connection with the several "strains" of rainbow trout mentioned in the literature, since some doubts must be entertained regarding an adequate biologic standardization of these "strains." These doubts are, in part, based on the fact that the rainbow trout belonging to a certain strain and grown in American hatcheries develop hepatomas, while the rainbow trout derived from eggs of the same strain but transferred many years ago to New Zealand and living there in a wild state, are apparently free from such tumors [Allen (*31*)].

These considerations on a genetic background of hepatomas in rainbow trout acquire additional importance because several reports on the occurrence of hepatomas in rainbow trout in this country and abroad date back about 30 years [Haddow and Blake (*32*); Wales (*33*); Ellis (*34*)]. Although these tumors are evidently not entirely of recent date, the impression nevertheless exists in certain quarters that there has been a distinct increase in frequency of hepatomas during recent years in Italy, France, and the United States [Cudkowicz and Scolari (*20*); Levaditi, Besse, Vibert, and Nazimoff (*21*)]. Some doubts must be entertained whether this "impression" accurately reflects actual developments, because during recent studies on the occurrence of hepatomas among rainbow trout it was found that a severe epidemic of this disease apparently had existed unnoticed for some time among the trout of several hatcheries and was only discovered in the course of systematic investigations for the presence of hepatomas recently made in all hatcheries. The time of onset of the present epidemic of hepatomas thus remains uncertain despite

the statement of Wood and Larson (*19*) that, according to their experience, the occurrence of such tumors in American rainbow trout was noted only during recent years.

It has been asserted, moreover, that for genetic reasons hepatomas do not occur among "wild" rainbow trout [Wales (*33*)]. The actual scientific value of such findings, however, is doubtful because many public and private waters for many years have been stocked at irregular intervals with rainbow trout raised in hatcheries. It is, therefore, uncertain whether any "wild" specimens carrying hepatomas are members or descendents of this group of cultured "wild trout." The presently available evidence suggests that genetic influences have, at best, a modifying effect on the cancer-inducing process in trout liver, but are not of primary causal nature.

Serious consideration has been given during the past year to the possibility that the hepatomas are of viral origin because of their almost general distribution among the stock of many hatcheries. The possible existence of a relationship between viral infections and hepatocarcinogenesis in man and birds has been an active issue due to the occasionally observed development of primary carcinomas of the liver in individuals suffering from the cirrhotic after-effects of viral infectious hepatitis and has been proposed as one of the main causes of the high incidence of primary liver cancer among the African Bantu [Steiner *et al.* (*35*); Ratcliffe (*36*)]. The merits of the viral hypothesis of hepatocarcinoma in trout are determined at present by the following observations: Livers with early lesions do not exhibit any histologic evidence of an inflammatory hepatitic nature. The cancer cells, moreover, do not contain any inclusion bodies. All attempts at a cell-free transfer of hepatoma extract have so far failed [Rucker (*37*); Cudkowicz and Scolari (*20*)]. The cultivation of a virus from extracts of tumor tissue inoculated into embryonated chicken eggs was unsuccessful [Wood and Larson (*19*)]. Fingerlings obtained from eggs of rainbow trout of an eastern hatchery, which in all probability contained a virus (if such an agent were responsible for causing hepatomas), developed into adult fish, of which those kept on a commercial, processed, and medicated feed had many hepatomas while those fed sheep liver remained free from such neoplasms. Brown and brook trout kept in the same troughs with rainbow trout that had large hepatomas were free from hepatic tumors and, therefore, did not acquire an effective virus infection through contact with the rainbow trout, if it is assumed that such an agent was present among the latter. Prolonged cohabitation of sick rainbow trout with healthy ones was likewise ineffective in transmitting the hypothetical hepatocarcinogenic agent [Cudkowicz and Scolari (*20*)]. The total evidence so far available, therefore, does not lend any substantial support to the viral theory of hepatoma formation in trout.

The greatest amount of attention has been given to the nutritional theory as a possible source of liver cancer [Thompson (*15*); Wood and Larson (*19*); Rucker, Yasutake, and Wolf (*16*)]. This theory is attractive for various reasons. Radical changes have occurred in fish culture

and nutrition since about 1930 when the diet, which was previously composed mainly or entirely of meat scraps, was increasingly replaced by a prepared feed containing considerable amounts of scrap marine fish, liver meals, and cereal grain products [Wood and Larson (*19*)]. Present-day trout feed, which was developed for the special purpose of promoting rapid growth and increased weight of the animals, consists of a great variety of calorie-providing constituents, vitamin nutrients, chemical additives, and frequently, also, medicinal agents. A typical feed thus may be composed of liquid fish solubles, herring fish meal, wheat mill run, alfalfa meal, dried blood meal, meat scrap, salmon oil, corn solubles and dried distillers' grains, whey, steamed bone meal, calcium carbonate, Norwegian kelp meal, cottonseed-oil meal, ascorbic acid, calcium pantothenate, *p*-aminobenzoic acid, inositol, methionine, vitamin A, vitamin D, vitamin E, niacin, pyridoxine, choline chloride, yeast, riboflavine, vitamin B_{12}, sodium chloride, butylated hydroxytoluene (antioxidant), carbarsone, sulfamerazine, and sulfamethazine.

According to past experimental experience, a carcinogenic effect on the liver from dietary sources may be exerted. This may be through the mechanism of a specific dietary deficiency such as choline deficiency, by the production of a nutritional imbalance such as that resulting from feeding bentonite, or by the oral or parenteral administration of various chemical carcinogens such as aramite, DDT, tannic acid, carbon tetrachloride, chloroform, thiourea, thiouracil, thioacetamide, acetamide, dimethylnitrosamine, aminoazotoluene, *p*-dimethylaminoazobenzene, safranol, or selenium.

Recent observations in man attest to the fact that the ingestion of inorganic arsenicals, used as pesticides, may result in the delayed appearance of liver carcinomas and sarcomas, as may the intravenous injection of collodial thorium dioxide (thorotrast) solution. On the other hand, it has become rather doubtful in recent years that conditions of prolonged starvation, such as those present particularly in kwashiorkor, may lead directly to the development of liver carcinoma in man; also, the high frequency of chronic starvation and dietary imbalance, particularly protein deficiency prevalent among Indians, is not associated with an especially high primary liver cancer rate, such as that existing under similar nutritional conditions in the Bantu. Observations made with the carcinogenic action of several azo dyes, however, indicate that liver cancers develop only in rats when they are kept on a riboflavine-deficient diet. Hepatic carcinogenesis, therefore, appears to be a rather complex process when occurring in mammals, and may at times be the result of a combined action of hepatocarcinogenic or merely hepatotoxic, exogenous, and endogenous agents.

Among the constituents of the trout feed listed, the sulfonamides, butylated hydroxytoluene, and carbarsone are recognized hepatotoxins, while carbarsone, an organic arsenical amebicide, may be a direct liver carcinogen. Since the subcutaneous administration of oxidized and polymerized vegetable oil into rats was followed by the development of sar-

comas at the site of injection, there is some chance that the fish oils, when subjected to high temperatures usually ranging from 150° to 180° C but sometimes as high as 700° C during the flame-drying processing of fish meal, undergo polymerization and oxidation so that carcinogenic conversion products are formed. It is also possible that fatty acids may be changed during the oxidation processes into peroxides and epoxides, some of which are known to be carcinogenic. It is not likely that rancid fats in the feed possess carcinogenic properties, since large population groups in southern and central Asiatic countries have consumed rancid butter in their daily diet for centuries without acquiring abnormally high liability to cancer of the liver.

While it is likely that the new commercial feeds may specifically be imbalanced quantitatively and qualitatively, there is not, so far, any valid evidence to incriminate such alterations in the production of liver cancers in trout. In this respect, special attention may be called to the destruction of sulfhydryl groups which results from the processing procedures used in the preparation of fish meals.

Despite the obvious uncertainty concerning the suspected principal causal role of recently introduced commercial feed in the production of liver carcinomas in trout, the nutritional theory is supported at present by the bulk of circumstantial evidence. In this study, such support is derived from the fact that in a comparison of groups of rainbow trout coming from the same egg supply, those that were fed commercial medicated feed in a hatchery developed hepatocarcinomas in practically 100 percent of the examined brood stock in the hatchery population, while those in the National Aquarium that were fed sheep liver remained free from liver tumors. Observations with similar connotations in regard to the importance of the type of commercial feeds in the development of liver cancer, the age at appearance of the first tumors, and their incidence rate among the trout population are available from other sources [Thompson (15)].

The demonstration of hepatocarcinomas in rainbow trout in epidemic-like proportions is of distinct importance for several reasons. A new and abundant biologic medium is now available for the scientific study of hepatocarcinogenesis, which may be useful in the screening of chemicals employed in the human economy for potential carcinogenic properties to mammals, especially man. The occurrence of a severe epidemic of liver cancer among rainbow trout reared in large numbers in private and governmental hatcheries for food purposes has serious financial implications because the sale of diseased animals for human consumption and their transport through State lines is forbidden by Federal laws. While it has been maintained by some groups that the eating of such fish would not represent any health hazard to the consumer, this assumption is not justified because of a distinct lack of practical and scientific experience in such matters, and especially because the causal agent of this neoplastic disease is unknown. The fact that at times carcinomatous involvement affects not only the liver and other internal organs not ordinarily used for

human food, but at times also the muscle tissue, provides an additional reason for caution against any laxity in the application and enforcement of existing laws concerning the prohibition of the sale and transport of living and dead whole, eviscerated, frozen, and smoked diseased animals intended for human consumption.

The observation of this epidemic of liver cancer among edible fish, possibly related to the recent introduction and use of processed and medicated commercial feed, provides an important and serious warning to all concerned with the maintenance and protection of human health to devote greatly increased attention and efforts to an effective control of the growing number and variety of environmental, industry-related carcinogens. If such preventive measures are not taken, the stage will be set at a progressive rate for the future occurrence of a similar disaster to the human population as that which has overcome the rainbow-trout population on a national scale.

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PLATES

PLATE 160

FIGURE 1.—Liver of rainbow trout, 4 to 5 years old, studded with small and large white medullary nodes of hepatocarcinoma.

FIGURE 2.—Liver of rainbow trout, 4 to 5 years old, with several large cystic, colloidal nodes of hepatocarcinoma.

FIGURE 3.—Rainbow trout of similar age with several large and small reddish and white nodes, the largest measuring 4 cm in diameter and having a red-and-white-mottled cut surface.

FIGURE 4.—Cut surface of liver showing large, well-circumscribed, white, medullary nodes of hepatocarcinoma.

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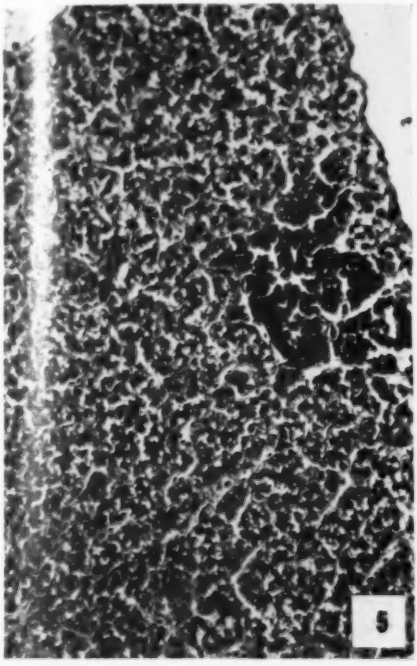
PLATE 161

FIGURE 5.—Ill-defined area of darkly stained and atypically arranged liver cells representing the primary focus of subsequent carcinomatous changes. × 143

FIGURE 6.—Small area of proliferating fibroblasts surrounding small groups of liver cells found in a liver with atypical liver cells similar to those shown in figure 5. × 143

FIGURE 7.—Large cellular hepatocarcinoma with large groups of carcinoma cells within the lumen of a hepatic vein. × 205

FIGURE 8.—Hepatocarcinoma with garland-like arrangement of carcinoma cells. × 143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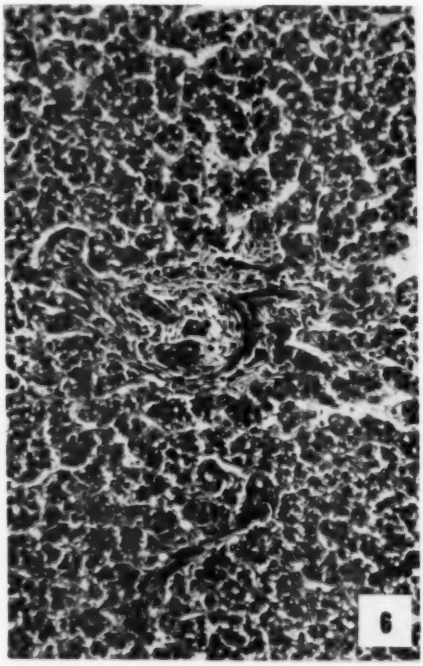
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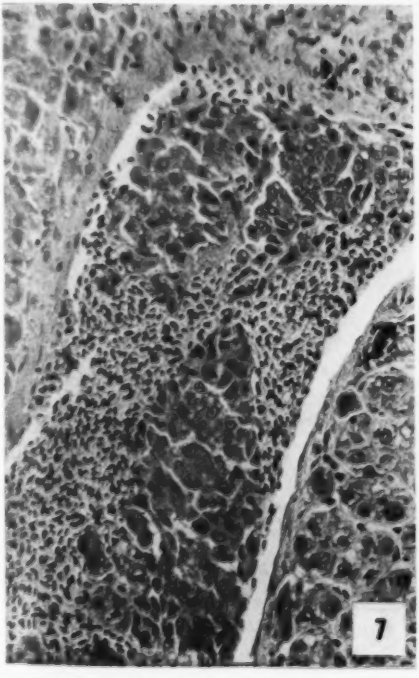
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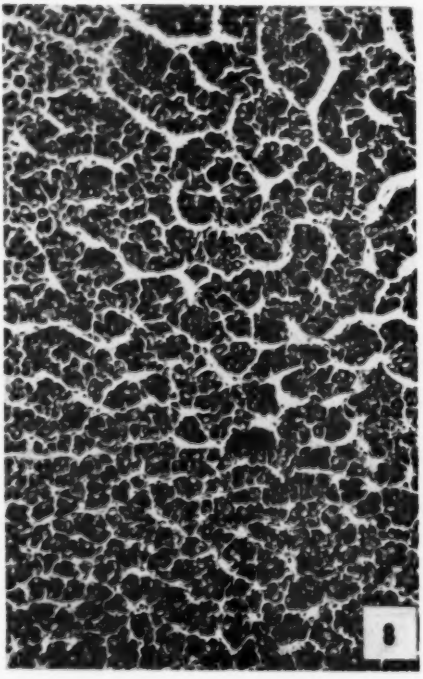
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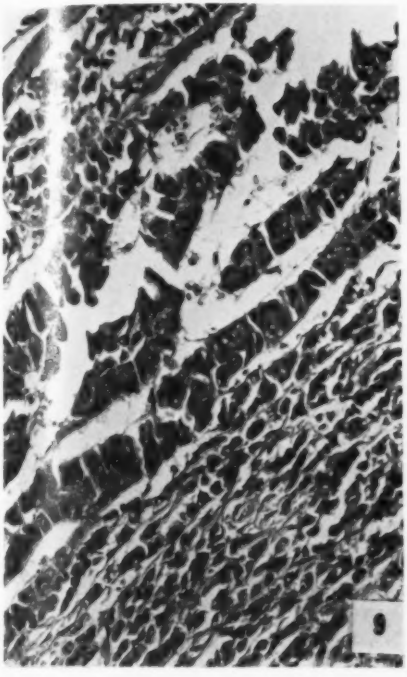
PLATE 162

FIGURE 9.—Glandular hepatocarcinoma surrounded by a small cellular sarcomatoid carcinoma. × 205

FIGURE 10.—Hepatocarcinomatous cell strands embedded in a fibrous stroma. × 143

FIGURE 11.—Groups and strands of pyknotic and hyperchromatic carcinoma cells surrounded by large masses of hyalinized stroma. × 205

FIGURE 12.—Hyalinized meshwork of hyalinized stroma with empty cavities indicating a "burned out" type of carcinomatous structure. × 143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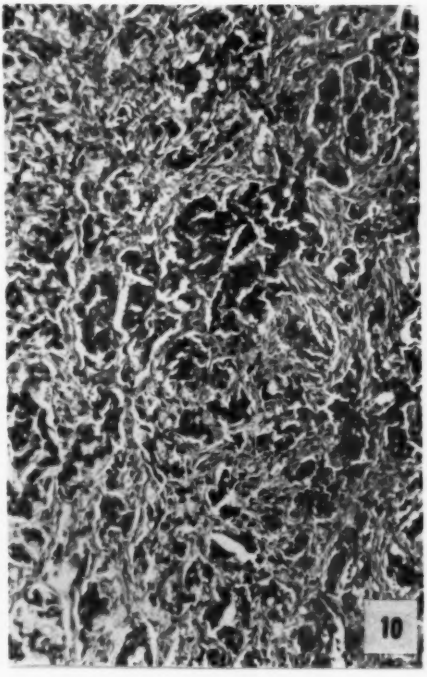
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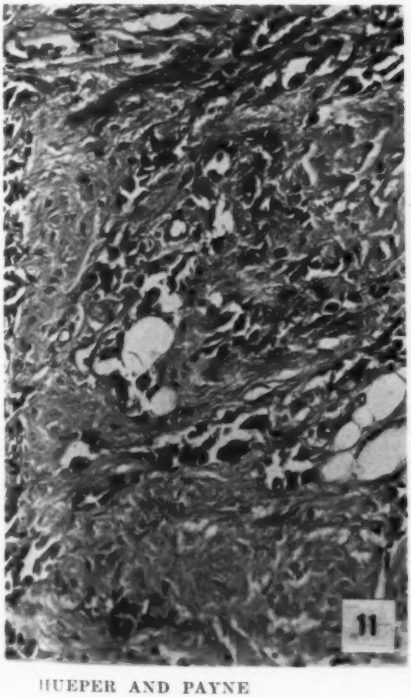
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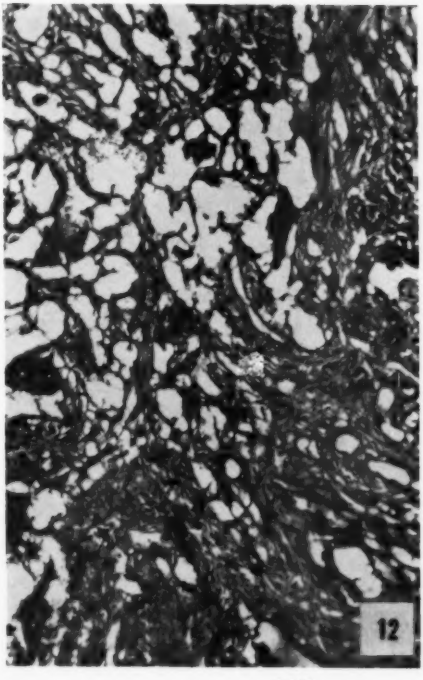
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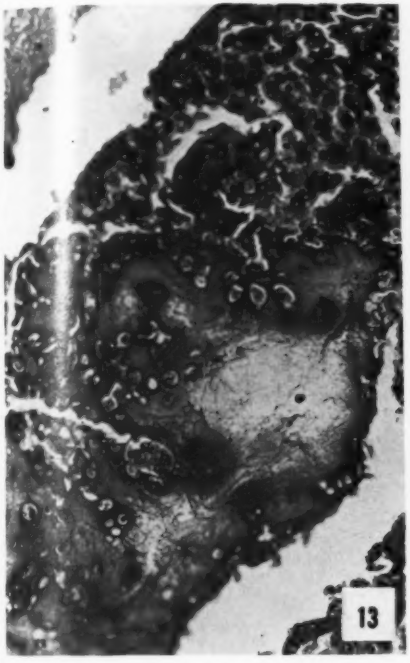
PLATE 163

FIGURE 13.—Hepatocarcinoma with "colloidal" degeneration resulting in formation of small and large "colloid" cysts. × 205

FIGURE 14.—Carcinoma of liver composed of a rather solid, small cellular hepatocarcinoma and of a cholangiocarcinoma. × 205

FIGURE 15.—Hepatocarcinoma invading skeletal muscle of anterior wall of body cavity. × 205

FIGURE 16.—Small group of hepatocarcinoma cells in spleen. × 205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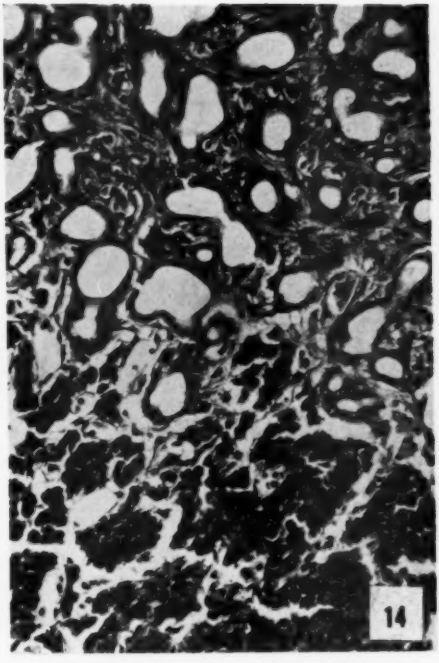
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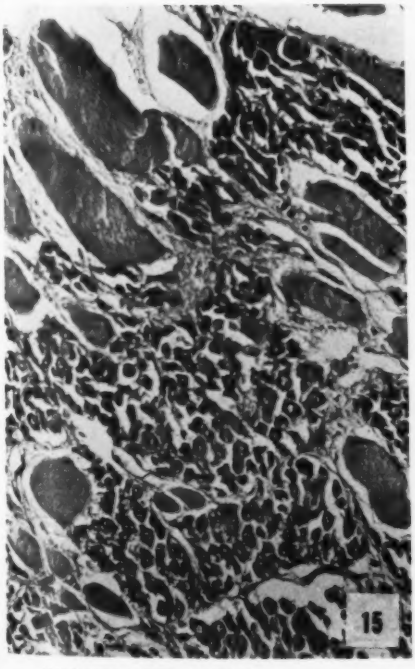
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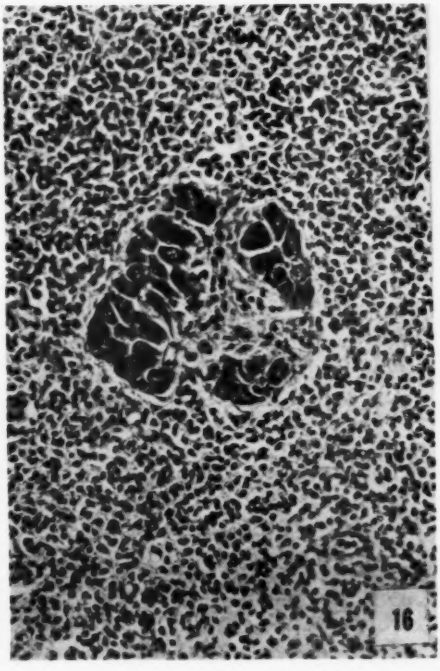
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Plasma Glycolytic, Citric Acid Cycle, and Transaminase Enzyme Levels in Hamsters With a Transplanted Mammary Adenocarcinoma [1, 2]

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SUMMARY

Plasma levels of glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), lactic dehydrogenase (LD), phosphohexose isomerase (PHI), aldolase (Ald), isocitric dehydrogenase (ICD), malic dehydrogenase (MD), and glutathione reductase (GR) were studied serially in hamsters for 30 days after transplantation of a nonmetastasizing mammary adenocarcinoma. The transaminase (GOT and GPT) levels remained essentially normal throughout the studies. The highest levels of the other enzymes were observed between the 22d and the 30th days. Values for the PHI and ICD showed the greatest degree of elevation, levels of the Ald and MD somewhat less marked elevations, while values of the LD and GR were least elevated. The highest plasma-enzyme levels were found when tumor mass was greatest, but tissue necrosis was also prominent. Nevertheless, distinctly elevated levels appeared even when necrosis was negligible.—J. Nat. Cancer Inst. 27: 1145–1152, 1961.

ANIMALS (or humans) bearing tumors have elevated levels of several plasma enzymes (*1–4*). Attention has been directed primarily to the enzymes of the glycolytic pathway as a presumed reflection of the increased glycolytic activity that uniformly characterizes tumor tissue (*5*). Studies in this laboratory revealed that patients with carcinoma of the breast (*6*) and gastrointestinal tract (*7*) have elevated levels of certain "oxidative" enzymes [isocitric dehydrogenase (ICD), malic dehydrogenase (MD), and glutathione reductase (GR)] as well as of those involved in glycolysis.

The levels of glycolytic enzymes in tumor-bearing animals apparently reflect the mass of the tumor (*2, 3, 8*). It is evident that, in patients with neoplasms metastasizing to distant sites, the mass of tumor cannot be quantitated satisfactorily. In this study, the plasma levels of "oxidative" and glycolytic enzymes in hamsters have been related to the size

[1] Received for publication June 15, 1961.

[2] This work was supported by a grant from the American Cancer Society, Inc., and a graduate training grant in Diseases of the Liver (2A–5225) of the National Institutes of Health, Public Health Service.

[3] Mount Sinai Hospital. Goldblatt Fellow for Cancer Research (supported by Goldblatt Brothers employees for the Nathan Goldblatt Cancer Research Fund).

[4] The authors wish to express their appreciation to Dr. Henry Rappaport, who reviewed the microscopic slides of the tumors.

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of a tumor that does not produce distant metastases. We are unaware of any studies comparing the values of "oxidative" and glycolytic enzymes of the plasma of tumor-bearing animals.

Increased levels in the plasma of lactic dehydrogenase (LD), aldolase (Ald), phosphohexose isomerase (PHI), MD, ICD, and GR were found in hamsters bearing a transplantable adenocarcinoma of the breast. The levels reached in the plasma appeared related partly to tumor mass and partly to the necrosis of the tumor, which increased as the tumor mass increased.

MATERIALS AND METHODS

Assay of the plasma enzymes listed in table 1 was performed by methods previously reported (*6*).

A total of 181 female, Syrian golden hamsters, 2 to 3 months of age, were studied. They were fed *ad libitum* with Purina laboratory chow. Heparinized blood was obtained for enzyme assay by cardiac puncture after the animals were anesthetized with 15 mg of Nembutal, and the animals were then killed. Samples with recognizable hemolysis were discarded. The enzyme values for normal hamsters (table 1) were determined by the study of 40 animals. The value shown for each enzyme is the mean of 8 determinations, each on the pooled plasma from 5 animals. In each of the 4 individual experiments, the number of animals indicated in table 1 received in the flank subcutaneous injections of 1 ml of a 10 percent suspension of mammary adenocarcinoma. This tumor, the biological characteristics of which are to be reported (*9*), does not produce distant metastases in hamsters, but grows locally to a large size and occasionally invades contiguous muscle. Assay of enzyme activity was performed on the pooled plasma from all the animals at the particular interval after transplantation, as indicated in table 1.

In 3 of the 4 experiments, the tumors were excised and weighed at the time the animals were killed. Microscopic sections of the tumors revealed an increasing amount of necrosis as the tumor increased in size. Examination of the carcasses failed to show gross metastases in the liver or other distant organs. The livers were also examined microscopically and failed to reveal malignant cells. Occasionally, invasion of muscle contiguous to the tumor was noted.

Experiments were conducted for only 30 days. Beyond this period, the tumor became so large that it produced necrosis of the overlying skin.

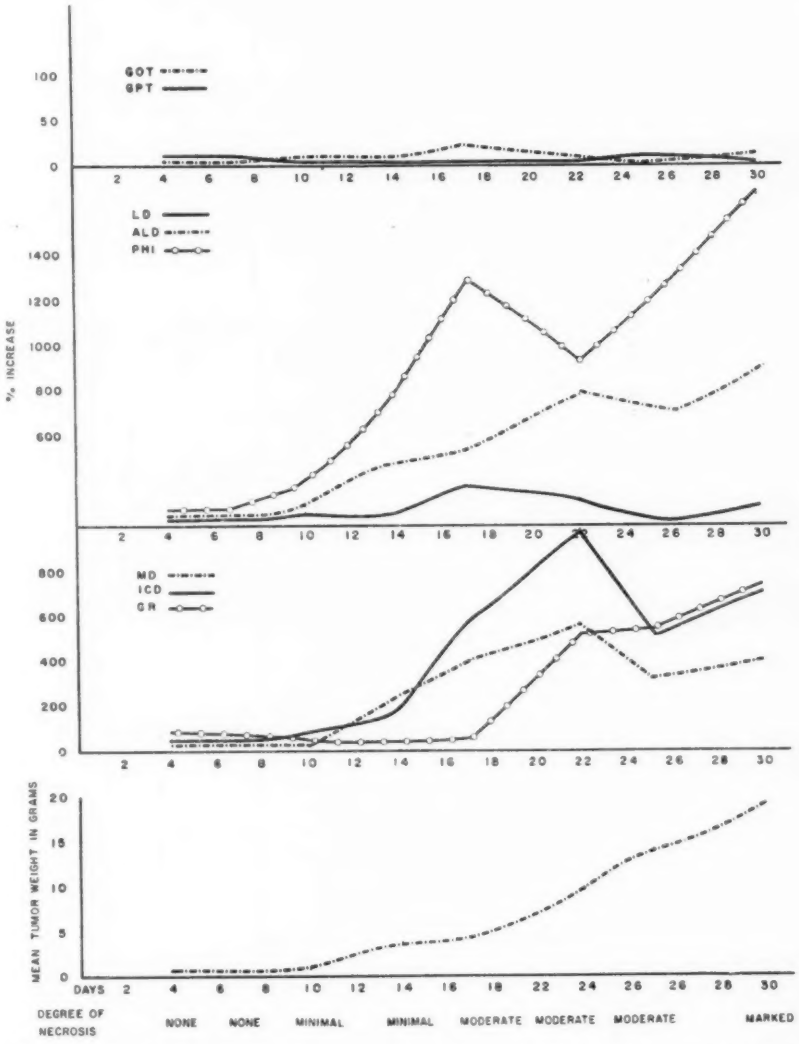
RESULTS

The levels of each of the enzymes studied, the weight of the tumor, and the histologic evaluation of the degree of necrosis at each stage of a rep-

resentative experiment (expt. III) are shown in text-figure 1. The results of the 4 experiments are summarized in table 1.

During the first 10 days, the tumor appeared to grow slowly and reached a mean weight of less than 1 g. Thereafter, growth was rapid and the tumor increased to 4 g by the 14th day, and to over 15 g by the 25th day.

Sections of the tumors, selected at random, showed little or no necrosis



TEXT-FIGURE 1.—Results of a characteristic experiment (III) showing the serial relationship of all plasma enzyme levels (in % increase above the normal mean) to mass of tumor and degree of necrosis.

TABLE 1.—Summary of results of four experiments

	Days after transplant	Number of animals	Glutamic oxaloacetic transaminase (Karmen units)	Glutamic pyruvic transaminase (Karmen units)	Lactic dehydrogenase (μM DPNH/100 ml/30 min)	Aldolase (mμM F-1, 6-P/ml/min)	Phosphohexose isomerase (mμM F-6-P/ml/min)	Malic dehydrogenase (mμM DPN/ml/min)	Isocitric dehydrogenase (mμM TPN/ml/min)	Glutathione reductase (Δ.001/0.D./ml/min)	Weight of tumor (g)
Enzyme plasma levels for 40 normal animals											
Mean			97	60	233	31. 5	981	830	41	52	
± 2 SD			151	92	365	48	1, 565	1, 144	93	128	
Range			66–148	41–80	168–331	22–47	577–1, 485	655–1, 125	9–74	20–140	
Enzyme plasma levels for tumor-bearing animals											
			Experiment I								
	4	6	82	22	309	27	591	475	28	95	0. 3
	7	6	150	54	408	39	872	855	48	78	0. 4
	14	6	79	24	455	27	1, 040	575	36	90	2. 2
	22	6	66	31	1, 260	125	10, 000	3, 750	315	167	14. 0
			Experiment II								
	7	6	83	18	309	31	1, 200	560	10	105	—
	10	6	95	69	547	43	1, 810	1, 480	17	78	—
	14	6	95	25	310	27	2, 135	675	90	90	—
	30	6	88	60	425	60	>3, 000	2, 725	150	46	—

Experiment III

4	10	93	38	298	14	856	750	32	51	0. 5
7	10	83	46	253	30	844	625	35	40	0. 5
10	8	113	48	300	55	1, 730	900	74	80	0. 8
14	8	128	42	274	90	5, 820	2, 100	185	66	4. 0
17	8	136	66	563	108	11, 200	3, 400	257	67	4. 0
22	9	103	41	450	182	7, 020	4, 530	396	276	9. 6
25	9	96	56	239	151	11, 140	3, 000	215	280	14. 0
30	15	117	27	333	227	14, 600	3, 417	280	387	19. 0

Experiment IV

15	8	128	34	515	85	2, 140	4, 250	575	183	5. 0
30	8	138	19	523	90	9, 280	2, 875	333	152	16. 0

during the first 2 weeks. During the 3d week, necrosis was classified as "moderate" (40 to 60 % of cells showing various degrees of necrosis), and by the end of the 4th week it was "marked" (60 to 80 % of the cells showing necrosis).

Mildly elevated levels of the 3 glycolytic and 3 "oxidative" enzymes were observed during the 2d week, and distinctly increased values of these enzymes were observed during the 3d and 4th weeks. The highest levels were observed between the 22d and the 30th days in all 4 experiments (table 1 and text-fig. 1). During this period, values for the PHI and ICD showed the greatest degree of elevation in the glycolytic and "oxidative" group, respectively. Levels of the Ald and MD showed somewhat less marked elevations, while values of the LD and the GR were least elevated. Transaminase values remained almost normal during all the experiments.

DISCUSSION

Our results agree with those of others (*2*, *3*, *8*) and show that animals bearing tumors have increased values of plasma glycolytic enzymes that appear to parallel the increasing size of the tumor.

A positive correlation between mass of tumor and degree of serum-enzyme elevation has been shown for Ald by Warburg and Christian (*1*), Sibley and his associates (*2*), and Mulay (*10*), for PHI and LD by Bodansky and Scholler (*3*), and for LD by Riley and Wroblewski (*8*). In the studies of Sibley and his co-workers (*2*), and of Bodansky and Scholler (*3*), elevated serum levels of the enzyme were noted only after increase in tumor size had been demonstrated, 1 to 2 weeks after transplantation. These results are comparable with those obtained in our study (text-fig. 1), but differ from the findings of Riley and Wroblewski (*8*), who reported a fourfold to twelvefold increase in serum LD prior to *detectable* growth of tumor implant.

Work from several laboratories (*2*, *3*, *10*, *11*) suggest that the elevated serum levels of the several glycolytic enzymes appear to reflect release from tumor tissue, which has a known high rate of glycolytic activity. Plasma levels of "oxidative" enzymes, such as MD, ICD, and GR, are also increased in tumor-bearing animals, as shown in the present study and in patients with carcinoma as reported elsewhere (*6*, *7*). Nevertheless, studies (*12*, *13*) on tumor-enzyme content reveal normal or depressed concentration of these "oxidative" enzymes.

The elevated levels of "oxidative" enzymes would suggest that factors other than abnormal glycolysis of tumors contribute to the occurrence of elevated enzyme levels in the plasma of tumor-bearing host. The increase in levels of both the "oxidative" and the glycolytic enzymes in parallel with the increase of the tumor mass suggest that entry into the serum of both groups of enzymes may depend on related mechanisms.

Increased release of these enzymes might reflect increased tumor content or altered cell-membrane permeability and/or tissue necrosis. In the studies of Mulay (*10*), Bodansky and Scholler (*3*), and Riley and Wroblewski (*8*), necrosis was considered negligible in the production of elevated serum levels of one or another of the glycolytic enzymes. Sibley *et al.* (*2*), however, considered necrosis of some importance. In our study the highest plasma-enzyme levels were found at the time of greatest tumor mass, when tissue necrosis also was most prominent. Nevertheless, distincly elevated levels appeared even when necrosis was negligible.

Other investigators (*14*, *15*) have reported that necrosis is not a prerequisite for enzyme release from neoplastic cells. Wu (*14*) has shown that glycolytic (and hexose shunt) enzymes may "leak" from apparently structurally intact tumor cells. Sylven and Bois (*15*) have presented evidence that neoplastic cells are characterized by increased permeability to some proteins including several enzymes.

The entry of a particular enzyme into the serum may partly reflect the intracellular concentration related to its metabolic role (*i.e.*, glycolysis). It may also depend on the physicochemical state of the enzyme within the cell, as well as on changes in cell membrane permeability and on frank necrosis. The effect of these several factors in leading to elevated plasma-enzyme levels may be expected to depend on *total* enzyme source (tumor mass and various organs).

Among the physicochemical factors that may be relevant is the intracellular location of the enzymes under consideration. It is reasonable to suppose that "soluble" (cytoplasmic) enzymes would gain entry into the plasma most readily. The 6 enzymes under consideration (Ald, PHI, LD, MD, ICD, and GR) are cytoplasmic enzymes (*16*). That factors other than the soluble state of the enzyme are important in leading to elevated plasma levels is suggested by the normal plasma values of the transaminases in this experiment, since these enzymes are also cytoplasmic (*16*).

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Long-Term Cultivation in Tissue Culture of Leukemic Cells From Mouse Leukemia Induced by Moloney Virus or by X Rays [1, 2]

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SUMMARY

Successful long-term *in vitro* cultures of leukemic cells have been obtained from BALB/c, C57BL/6, SWR, and Swiss mice, with leukemia induced by Moloney virus; and from C57BL/6 mice, with leukemia induced by X rays. The leukemic cells were grown from 4 to 10 months *in vitro*. All the cell lines were successfully maintained on feeder layers of normal mouse spleen cells in Eagle's medium supplemented with horse serum. Under these conditions the leukemic cells grew free in the medium, so that they could be readily passaged without trypsinization. The leukemic nature of the cultured cells was established by examination of cell smears and by cell transplantation into animals.—J. Nat. Cancer Inst. 27: 1153–1171, 1961.

SINCE THE original finding by Gross on the induction of leukemia by cell-free filtrates from leukemic AK mice (*1*), several murine leukemia viruses have been isolated (*2–5*). Leukemia-inducing activity has also been reported with extracts from X-ray-induced mouse leukemia (*6, 7*). In order to study, at the cellular level, the mechanism of the neoplastic transformation produced by treatment with X rays or leukemia virus, it is desirable to develop a system for the *in vitro* transformation of normal to leukemic cells. A basic requirement for such a system is the development of a simple method for the regular establishment of *in vitro* cultures of leukemic cells.

Bichel (*8*) and De Bruyn *et al.* (*9*) grew mouse leukemic cells in tissue culture by using fibroblasts as feeder layers, and more recently Brooke and Osgood (*10*) reported the growth of several types of human leukemic cells, which had not been cultivated previously, by using established cell lines as feeder layers (*11*). The culture of mouse leukemic cells without a feeder layer has been described by Fischer (*12*), who found it necessary to supplement the tissue-culture medium with a high level of folic acid and peptone. However, Fischer also noted that there were substantial differences in the nutritional requirements of different cell lines. With

[1] Received June 19, 1961.

[2] This work was supported by grant DRG 616 from the Damon Runyon Memorial Fund for Cancer Research.

[3] We are indebted to Mr. Aryeh Goldenberg for technical assistance.

chickens, Beaudreau *et al.* (*13*) grew long-term cultures of myeloblasts, induced by myeloblastosis virus, in medium with additional B vitamins, folic acid, and glucose.

The present paper reports a simple method for the establishment and maintenance of mouse leukemic cells by the use of feeder layers of normal mouse spleen cells. Seven lines of leukemic cells induced by Moloney virus (*5*) or X rays have been established by this method, and in all cases the cells grew free in the culture medium so that they could be readily passaged without trypsinization. Data on the suitability of cultures of lines of leukemic cells induced by Moloney virus (MLV), as a good and regular source of virus, have previously been reported (*14*, *15*).

MATERIALS AND METHODS

Induction of leukemia.—Leukemias were induced with MLV by subcutaneous or intraperitoneal inoculation of 0.05 ml of virus suspension into 3- to 12-day-old mice belonging to strains C57BL/6, SWR, DBA/2, or Swiss (noninbred), or by intraperitoneal inoculation of 0.2 ml into adult mice of strain BALB/c. For the induction of leukemia by X rays, 1- to 2-month-old C57BL/6 mice were irradiated once a week either with 4 doses of 170 r or 7 doses of 112 r. Transplants of leukemic cells were made by intraperitoneal inoculation into adult mice. All the leukemic mice had lymphatic leukemia, except for 1 mouse with chloroleukemia that was used to establish line MOL48.

Preparation of leukemic cells for culture.—Mice with primary or transplanted leukemia were opened under sterile conditions, and the organs used for the establishment of the cultures were removed to a petri dish containing phosphate-buffered saline, and washed 3 times. The organs were then transferred to a petri dish with culture fluid and teased into small pieces with forceps so as to release the cells into the medium. The medium with the suspended cells was then collected, allowed to stand for several minutes to sediment the large pieces, and the supernatant diluted to the required cell number in new medium. In most cases the cultures were established by seeding 1×10^7 to 2.5×10^7 cells in 50 mm petri dishes containing 4 to 5 ml of medium. All cultures were made and maintained in petri dishes kept at $36° C \pm 0.5° C$ in a humidified incubator with a constant flow of 10 percent CO_2 in air.

Medium, medium change, and passage of established cultures.—Unless otherwise stated, the culture medium consisted of Eagle's medium containing a fourfold concentration of amino acids and vitamins and supplemented with 20 percent horse serum. This is our standard medium. The maintenance of heavily populated cultures required a medium change every other day to reduce the cell number sufficiently to prevent degeneration; less populated cultures could be maintained satisfactorily by changing the medium twice a week. The medium change, which included a reduction in cell population, was made by removing the old medium with sus-

pended cells, except for 1 to 3 drops that contained sufficient cells for further proliferation, and then adding fresh medium. Since the cells grew free in the medium they could be passaged without trypsinization, and cells of established cultures were passaged by the transfer of 0.5 to 1 ml of cell suspension containing 2.5 to 5 $\times$ 10^6 cells to a new petri dish with a feeder layer of normal spleen cells.

Preparation of spleen feeder layer.—Suspensions of spleen cells from normal adult C57BL/6 mice were prepared in the same way as the organs of leukemic mice, and 2 to 3 $\times$ 10^7 cells were seeded with 4 ml of our standard medium in 50 mm petri dishes. After 5 to 10 days an optimal number of cells had stuck and spread on the glass, though there were still many cells free in the medium. The medium with the free cells was removed, the cultures were washed, new medium was added, and the plates were then ready for use as feeder layers. A reserve of such plates could be kept for many weeks by changing the medium every 7 to 10 days.

Preparation of cell smears.—During the preparation of cell smears for study, it was found that smears made from cells suspended only in horse serum were better than those made in the usual culture medium or in saline. As a routine procedure, the cells with the culture medium were therefore sedimented by low-speed centrifugation and the medium was discarded; the cells were washed once in horse serum and then resuspended in horse serum for smearing. The smears were made in the usual way, allowed to dry in air, and then stained with May-Grünwald-Giemsa.

Coverglasses were introduced into the petri dish for the examination of cells that stuck to the glass. The coverglasses were then washed, fixed with methanol, and stained.

EXPERIMENTS

Establishment of Long-Term Cultures of Cells

Ten experiments were carried out in the *in vitro* establishment of lines of leukemic cells, and the results are summarized in table 1. Eight of these were with cells from mice with leukemia induced by MLV, and 2 from mice with leukemia induced by X rays. The experiments, 7 of which resulted in the successful establishment of lines of leukemic cells, included the use of primary or transplanted leukemic cells, different strains of mice, different organs, different media, and different cell inoculums. After establishment, all the cell lines were regularly passaged on feeder layers of normal spleen cells, usually in Eagle's medium plus 20 percent horse serum.

For the establishment of MOL9, the following media were tested:

(*a*) 199 with additional B vitamins, folic acid, and glucose, according to Beaudreau *et al.* (*13*) for avian myeloblasts, plus 50 percent normal mouse serum
(*b*) 199 as in (*a*) plus 50 percent horse serum
(*c*) Eagle's plus 50 percent horse serum

TABLE 1.—*In vitro* establishment of lines of mouse leukemic cells

Name and No. of experiment	Strain of donor mice	Sex of donor mice	Origin of leukemia	Medium *	Organs used for cultures of leukemic cells	Number of cells seeded	Lag period (days)	Establishment of line of leukemic cells†	Duration of growth of cell lines *in vitro* (days)
MOL9	SWR	♂	MLV, first-transplant generation	EM + 50% HoS ‡	Spleen	2.5×10^7	24	+	154§
MOL10	C57BL/6	♂	MLV, primary leukemia	EM with 0.001% folic acid and 0.06% peptone + 10% HoS‡	Spleen	2.5×10^7	10	+	121§
MOL26	Swiss	♂, ♀	MLV, pool of 2 primary leukemias	EM + 20% HoS	Spleen, lymph nodes, thymus, and kidney	1.6×10^7	7	+	320
MOL48	Swiss	♂	MLV, primary chloroleukemia	EM + 20% HoS	Spleen and lymph nodes	1 and 2×10^7	0	+	173
MOL50	BALB/c	♀	MLV, primary leukemia	EM + 20% HoS	Lymph nodes and thymus	1×10^3, 10^3, 10^4, 10^5, 1×10^6, 1×10^7	— 10	— +	 164
RL30	C57BL/6	♂	X rays, pool of 2 from eleventh-transplant generation	EM + 20% HoS	Spleen and lymph nodes	1×10^7 2×10^7	9 —	+ —	177§
RL31	C57BL/6	♂	X rays, primary leukemia	EM + 20% HoS	Spleen, lymph nodes, thymus, and kidney	1×10^7 2×10^7	7 4	+ +	 270
MOL29	DBA/2	♂	MLV, primary leukemia or transplant ‖	EM + 20% HoS	Spleen and lymph nodes	2×10^7	—	—	—
MOL32¶	C57BL/6	♂	MLV, tenth-transplant generation	EM + 20% HoS	Spleen, lymph nodes, and kidney	1 and 2×10^7	—	—	—
MOL43	Swiss	♂, ♀	MLV, pool of 8 primary leukemias	EM + 20% HoS	Spleen, lymph nodes, and thymus	1, 2, 3, and 4×10^7	—	—	—

* EM = Eagle's medium. HoS = horse serum.

† + = Establishment of line, successful; — = establishment of line, unsuccessful.

‡ The medium that resulted in the establishment of a cell line.

§ Time when cell line discarded.

‖ The donor mouse received injections of cells; it is not known whether the leukemia developed as the result of virus induction or growth of the inoculated cells.

¶ Tenth-transplant generation of the primary leukemia serving for the establishment of MOL10.

There was complete degeneration of cells in the cultures with normal mouse serum. The cultures with the other two media survived for some time, with approximately 1×10^6 of a variety of cell types per petri dish. These cultures at first resembled those of normal spleen, until, in 1 of the 8 petri dishes with Eagle's medium, leukemic cells proliferated on day 24. The cells from this plate were then passaged in Eagle's medium plus 50 percent horse serum on feeder layers of normal spleen cells.

The following media were tested for the establishment of MOL10:

(*a*) 199 as in (*a*) for MOL9 plus 50 percent normal mouse serum
(*b*) 199 as in (*a*) plus 50 percent horse serum
(*c*) 199 as in (*a*) with 0.001 percent folic acid plus 50 percent horse serum
(*d*) 0.5 percent lactalbumin hydrolysate in Earle's saline with 0.001 percent folic acid plus 0.06 percent peptone plus 20 percent horse serum [folic acid and peptone concentration according to Fischer (*12*)]
(*e*) Eagle's with 0.001 percent folic acid plus 0.06 percent peptone plus 10 percent horse serum

Only medium (*e*) gave successful growth, and foci of proliferating leukemic cells were recognized, after 10 days, in all 6 petri dishes seeded in this medium. On day 10, this medium was replaced by the standard Eagle's medium plus 50 percent horse serum.

The cells used for the establishment of MOL26 were seeded in Eagle's medium plus 20 percent horse serum, either in clean petri dishes or on mouse-embryo monolayers, and proliferation was observed in both sets of cultures at 7 days.

The cells for MOL48 were taken from the green lymph nodes and red spleen of a mouse with primary chloroleukemia. In contrast to the other experiments, the cultures remained well populated from the beginning and there was no initial cell degeneration. The bottoms of the petri dishes were rapidly covered with a considerable number of large, round cells. It was necessary to reduce the cell population at 3 days after the initial seeding, and a burst of cell proliferation was observed at 14 days.

For MOL50, tenfold dilutions from 1×10^2 to 1×10^7 cells from a pool of thymus and mesenteric lymph-node cells were seeded on mouse-embryo monolayers, and 1×10^7 or 2×10^7 cells were seeded on empty petri dishes with Eagle's medium plus 20 percent horse serum. Pronounced degeneration occurred in all the dishes, and 1 to 4 foci of leukemic cells per plate were found (at 10 days) only in the plates seeded with 1×10^6 or 1×10^7 cells on mouse-embryo monolayers. Since these cell colonies stuck to the embryo feeder layer, the cells were dispersed by trypsin and transferred to new plates either with or without new embryo feeder layers. Both sets of cultures grew well.

In RL30, a complete degeneration occurred in the plates seeded with 2×10^7 cells, and in this group there were only small foci of leukemic cells (in 2 of 8 plates) that were phagocytosed after a few days. But cell proliferation was found at 9 days, in 6 of 8 plates seeded with 1×10^7 cells. In RL31, early cell proliferation was more pronounced in the cultures seeded with 2×10^7 cells.

The experiments MOL29, 32, and 43 did not result in the *in vitro* establishment of lines of leukemic cells. The cultures were kept for 25 to 41 days and then discarded, since all the free cells had gradually disappeared.

General Behavior of Cultures During the Establishment of Cell Lines

During the first days after seeding there was usually considerable cell death. This was then followed either by complete degeneration of the leukemic cells or, when cell lines were successfully established, by the proliferation of foci of surviving leukemic cells. Many apparently normal cells could be seen during the course of the leukemic cell degeneration. Most of these were either histiocytes or fibroblast-like cells that stuck to the glass. In addition, there were cells of the granulocytic series in various stages of maturation, plasma cells, megakaryocytes, normoblasts, lymphocytes, and other unidentified cells that remained free in the medium. When the leukemic cells completely degenerated, the cultures attained a period of stabilization with 1 to 2 $\times$ 10^6 free, presumably normal, cells. The number of these free cells was gradually reduced during the first 2 to 4 weeks because of maturation followed by degeneration, and eventually the only surviving cells were those sticking to the bottom of the petri dish. Such cultures then resembled those of normal spleen.

When leukemic cells survived and proliferated, a heavily populated culture of free cells was obtained. The sticking cells that appeared earlier remained and served as a feeder layer. MOL9 was exceptional in that the proliferation of leukemic cells was observed only at 24 days after seeding, *i.e.*, much later in the development of the culture than in the other cases of proliferation. In the early stages of proliferation, the leukemic cells were generally found in small clusters in the vicinity of large, round cells, often forming a rosette. The proliferating leukemic cells did not stick to the glass, but floated freely in the medium. They also stuck only to a small extent to the feeder layer of normal mouse-spleen cells, though they soon stuck to the feeder layer of normal mouse-embryo cells.

With spleen feeder layers, an average of approximately 3 to 3.5 $\times$ 10^7 leukemic cells could be regularly harvested from a 50 mm petri dish. The population could be increased to approximately 4.5 $\times$ 10^7 cells for a short period, but such a dense cell population had to be quickly reduced since it led to rapid degeneration. Although no accurate cell growth curves have as yet been determined, approximately 4 $\times$ 10^7 cells could be collected 3 to 4 days after seeding 5 $\times$ 10^6 cells.

The Feeder Layer

For routine passaging, spleen feeder layers were preferred to mouse-embryo feeders, because the leukemic cells did not stick to any appreciable extent to the spleen feeder layer and they could thus be readily passaged without trypsinization. When embryo feeder layers were used, the

leukemic cells stuck to the feeder and formed dense, localized regions of leukemic cells that were so crowded that apparently only the outer cells survived. There was also a rapid drop of *p*H that seemed associated with further cell death. The life of such cultures was short, and it was necessary to make frequent cell passages in addition to the inconvenience of trypsinizing the cultures for passaging.

With the spleen feeder layer, the cultures kept for a long time, depending on the cell population. With a cell population of 1 to 1.5 $\times$ 10^7 cells per plate, the cultures could be kept for 2 to 3 months. Only a light seeding of spleen cells sparsely scattered on the bottom of the petri dishes was sufficient and no irradiation was necessary. Owing to its phagocytosing ability, the spleen feeder also disposed of dead cells and debris. Although the spleen feeder proved very satisfactory for the maintenance of large populations of leukemic cells, embryo feeders proved useful, despite the inconvenience of trypsinization, for cloning of small populations, since they do not seem to have the same phagocytosing ability as the spleen feeder. Cell populations could to some extent be maintained in a proliferating state without a feeder layer, but there was always a high proportion of dead cells with those that survived.

Appearance of Cultured Leukemic Cells

When examined in Giemsa smears (figs. 7 through 10), the cells of the lines appeared similar to the leukemic cells found in the blood and organs taken from animals. They had a thin or medium-sized, blue, nongranular cytoplasm, with a large, round, or indented nucleus with coarse fibrous chromatin, and generally 1 or 2 or sometimes 3 or 4 nucleoli. A chromophobic region could be seen near the nucleus. In MOL48, the cells were slightly larger, the nucleus was more granular and lumpy, the nucleoli were more pronounced, and the cytoplasm was wider with a chromophobic perinuclear region. Examination of the living cultures showed that there were differences in cell shape that could be used to characterize the cell lines. Thus, *e.g.*, the cells of MOL10 usually had a pyriform shape, those of MOL26 generally had one cytoplasmic process (fig. 2), those of MOL50 had many such cytoplasmic processes, and the cells of RL30 and RL31 (fig. 1) were round without cytoplasmic processes.

Living cells in the lines MOL26, MOL48, MOL50, RL30, and RL31 were observed with a phase-contrast microscope. Cells from RL30, RL31, and MOL26 are shown in figures 3 through 6. The cytoplasmic membrane in cells of the X-ray-induced leukemias (RL30 and RL31) is smooth and regular. However, in MOL26, derived from a leukemia induced by MLV, cells have been observed with an unusual appendage, 1 per cell, that may appear in 2 forms (figs. 5 and 6). The first form appears as a rough, beardlike protrusion (figs. 5 and 6, *B*), whereas the second appears as an indented protrusion (figs. 5 and 6, *A*). In one preparation of MOL26, chosen at random, 30 percent of the cells showed the first form, 40 percent the second form, and 30 percent were without

such an appendage. Observations on MOL48 and MOL50 showed no such appendages to the cells, and cells derived directly from a mouse with MLV-induced leukemia also did not show this peculiarity. The nature of this unusual appendage in MOL26 is not yet known.

Transplantation of Cultured Leukemia Cells Into Mice

The leukemic nature of the cultured cells was determined both by microscopic examination of the cells and by cell transplantation tests in animals (table 2). The results in table 2 show that of 5 lines originating from cells from inbred strains of mice, successful transplants were readily obtained with MOL9, MOL50, RL30, and RL31. The low degree of transplantability of the cultured cells of MOL10 apparently is not the result of culture *in vitro*, since it was also difficult to transplant cells used for the establishment of this line taken straight from the mouse (table 2). The cells of MOL26 originated from leukemia in 2 noninbred Swiss mice, so that it is not surprising that the cell-transplantation tests with this line in adult mice were unsuccessful.

DISCUSSION

Since the purpose of the present study was to develop a simple method for the successful *in vitro* cultivation of mouse leukemic cells, two aspects of the behavior of these cells must be considered. The first concerns the conditions for the original establishment of leukemic cells in culture, and the second the conditions for the maintenance of the cell lines. The present experiments showed that during the first few days after seeding *in vitro*, the cultures advanced toward a crisis with considerable cell degeneration. In cultures that overcame this crisis, there was then a proliferation of foci of surviving cells. Once this proliferative stage had been reached, there was no difficulty in maintaining the cultures with a high level of multiplication on spleen feeder layers in Eagle's medium plus horse serum.

Out of the 10 experiments, 8 with leukemia induced by MLV and 2 with leukemia induced by X rays, 7 resulted in the successful establishment of *in vitro* cell lines, whereas 3 were unsuccessful. Although there was no difficulty in maintaining the cell lines, there were some so far unpredictable factors that governed the ability of the cultures to overcome the the initial crisis. Further experiments with different media are therefore necessary to permit a regularly predictable control over the crisis period. Since mouse-embryo feeder layers were useful for cloning of leukemic cells, this type of feeder layer may also be useful for the original establishment of the cells *in vitro*.

Despite the drawback of this lack of an absolute control over the crisis period, the present results show that the use of spleen feeder layers and Eagle's medium with horse serum provides a simple working system for

TABLE 2.—Development of leukemia in mice inoculated with cells from the *in vitro* lines

Cell line	Time in culture (days)	Receptor mice	Age of receptor mice (months)	Number of cells inoculated	Number of mice that died with leukemia / Number of mice inoculated	Average time to death (days)
MOL9	24	SWR	2.0	1×10^5	2/2	27
MOL9	46	SWR	2.0	7×10^6	5/5	12
MOL10	0*	C57BL/6	1.0	Pieces of leukemic tissue	3/5	34
MOL10	0*	C57BL/6	4.0	Pieces of leukemic tissue	0/5	—
MOL10	45	C57BL/6	4.5	5.4×10^6	0/2	—
MOL10	45	SWR	1.5	5.4×10^6	0/5	—
MOL10	49	C57BL/6	2.5	5×10^6	2/13	36
MOL26†	22	Swiss	2.0	4×10^6	0/5	—
MOL26†	22	SWR	2.5	3×10^6	1/5‡	126‡
MOL50	56	BALB/c	5.0	9×10^6	2/2	46
RL30	16	C57BL/6	1.0	9×10^6	5/5	16
RL30	160	C57BL/6	1.0	5.5×10^7	5/5	19
RL31	70	C57BL/6	1.0 and 1.5	1.6×10^7	5/5	26
RL31	157	C57BL/6	1.0	7.2×10^7	5/5	32

*Cells transplanted from donor to receptor mice without *in vitro* culture.
†This line originated from cells from noninbred Swiss mice.
‡The leukemia in this mouse was presumably induced by virus.

the *in vitro* establishment and maintenance of mouse leukemia cells induced by leukemia virus and by X rays. The free growth of the cells in the medium under these conditions also provides a ready source of suspended cells that can be used for quantitative studies.

Since cultures of lines of leukemic cells induced by MLV are a good source of virus (*14*, *15*), the lines of cells from X-ray-induced leukemia are also being tested for the presence of leukemia virus. The *in vitro* culture of leukemic cells may thus prove to be a generally useful method for the isolation of leukemia viruses. In addition, the culture system used in the present experiments would seem to be of potential value for studies on the *in vitro* transformation of normal cells by mammalian leukemia viruses, similar to those undertaken with avian myeloblastosis (*16*) and polyoma (*17*, *18*).

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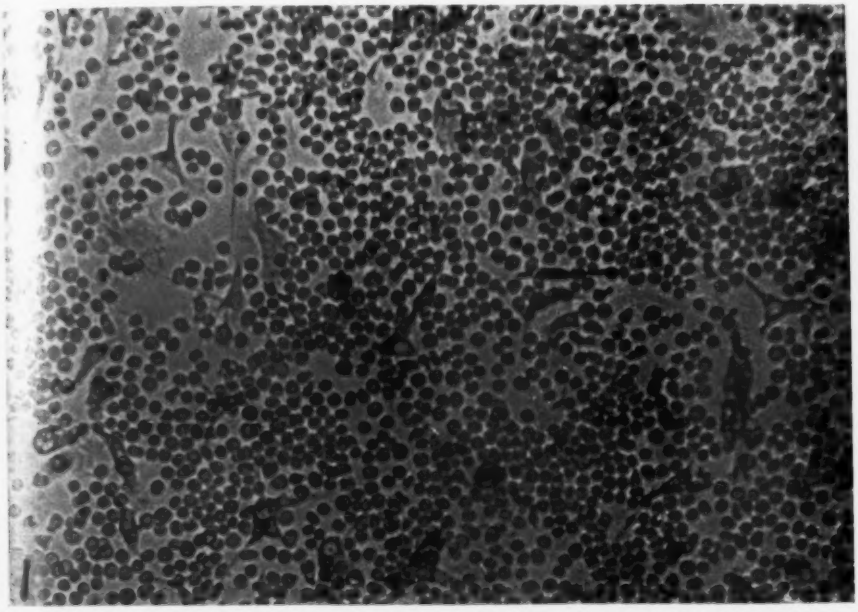
PLATE 164

FIGURE 1.—General view of a living culture of leukemic cells from RL31 after 106 days *in vitro*. Fibroblast-like cells are from feeder layer of normal spleen cells. × 200

FIGURE 2.—General view of a living culture of leukemic cells from MOL26 after 153 days *in vitro*. Fibroblast-like cells are from feeder layer of normal spleen cells. × 200



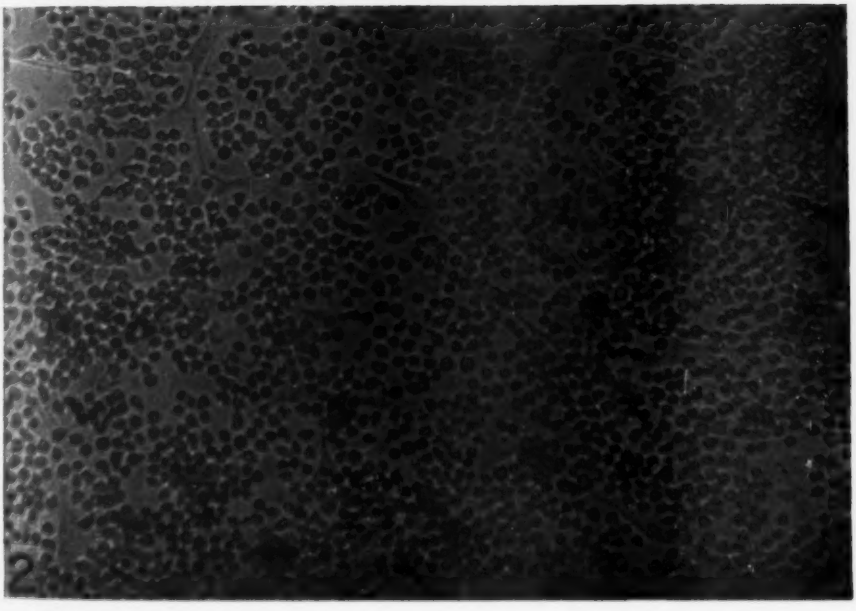
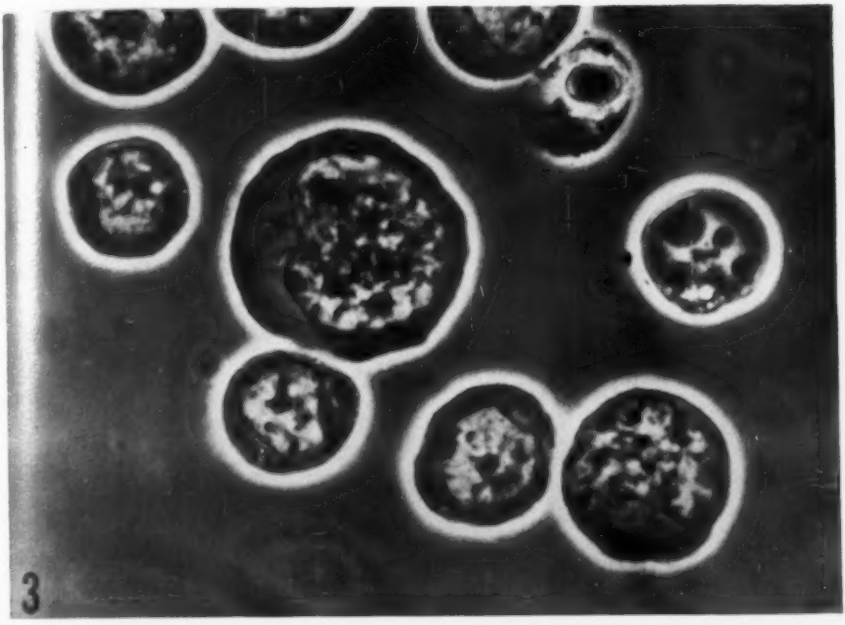


PLATE 165

FIGURE 3.—Phase-contrast photograph of living cells of RL30 after 116 days *in vitro*. × 2,500

FIGURE 4.—Phase-contrast photograph of living cells of RL31 after 113 days *in vitro*. × 2,500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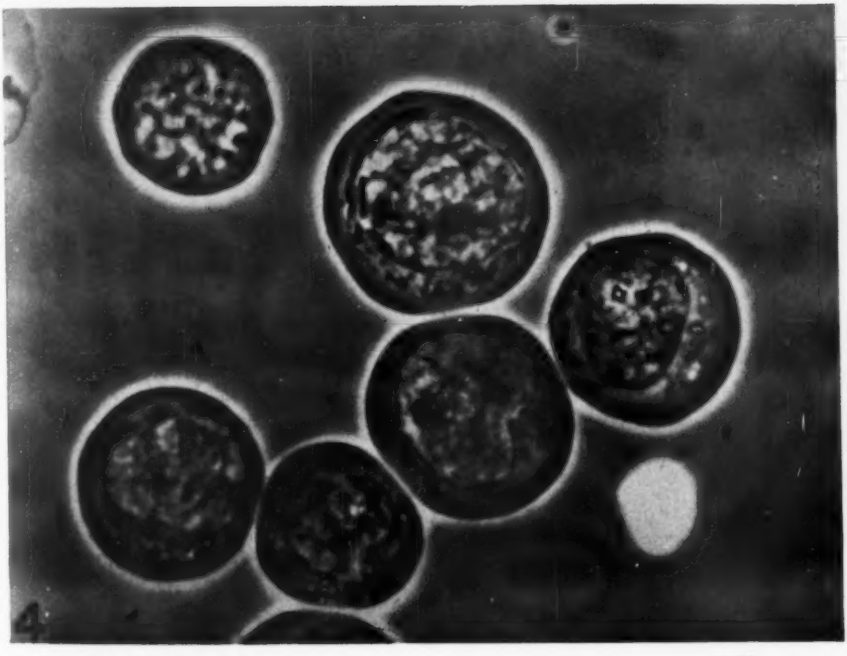
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PLATE 166

FIGURES 5 AND 6.—Phase-contrast photographs of living cells of MOL26 after 153 and 161 days *in vitro*. Examples of unusual appendages found in cells of this line (*arrows*). *A* = indented protrusion; *B* = beardlike protrusion. × 2,500

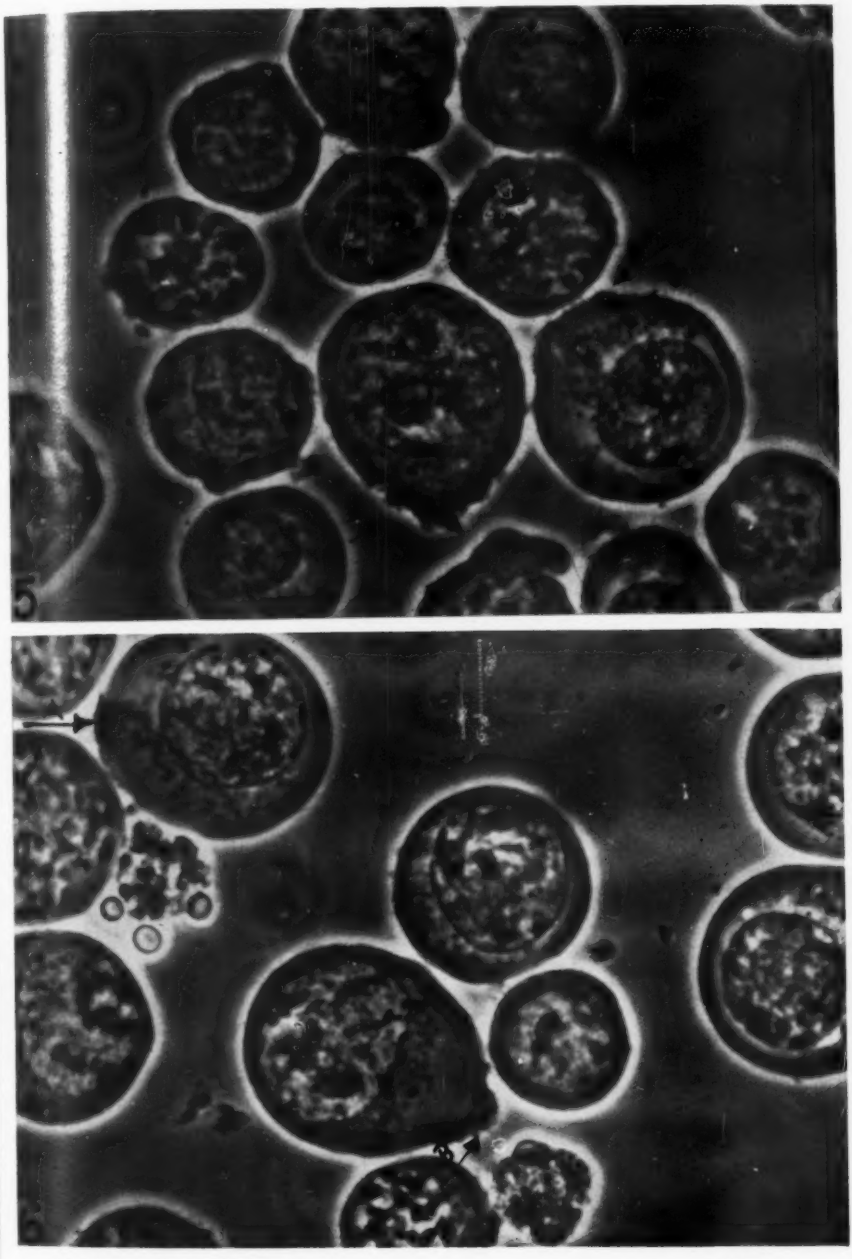

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PLATE 167

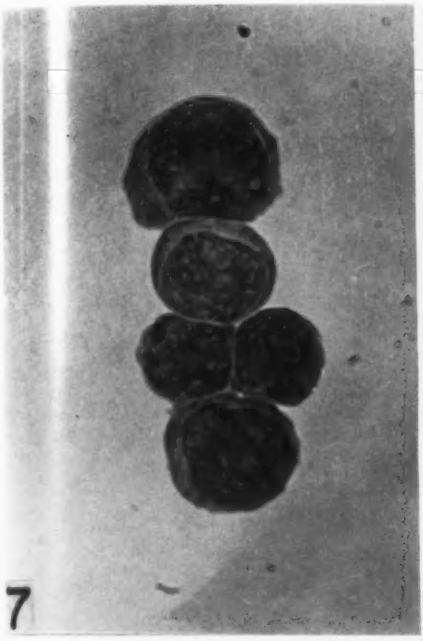
May-Grünwald-Giemsa smears of leukemic cells from the *in vitro* lines. × 1,400

FIGURE 7.—RL31 after 30 days *in vitro.*

FIGURE 8.—MOL48 after 30 days *in vitro.*

FIGURE 9.—MOL26 after 206 days *in vitro.*

FIGURE 10.—MOL26 after 35 days *in vitro.*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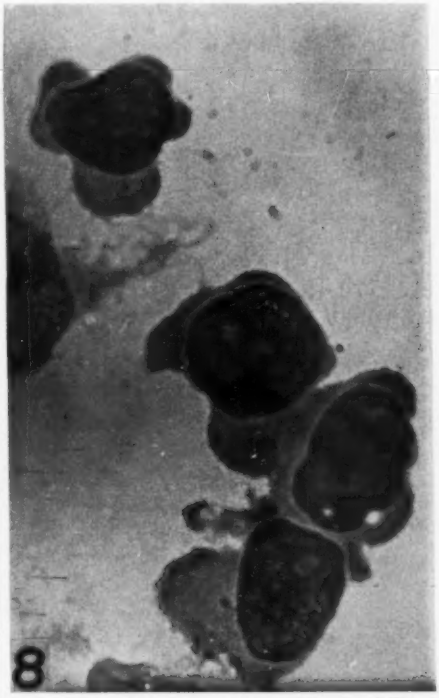
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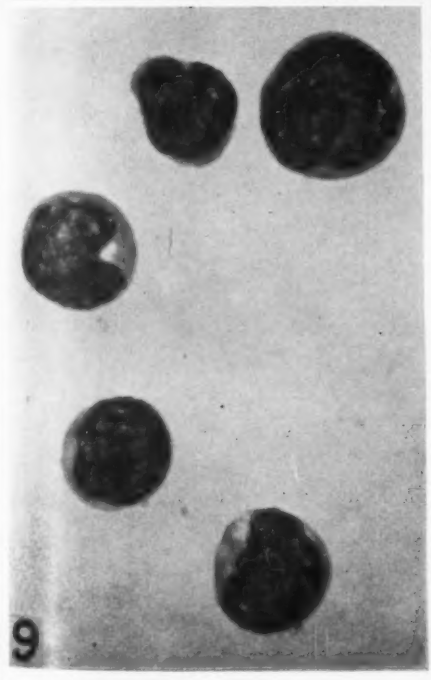
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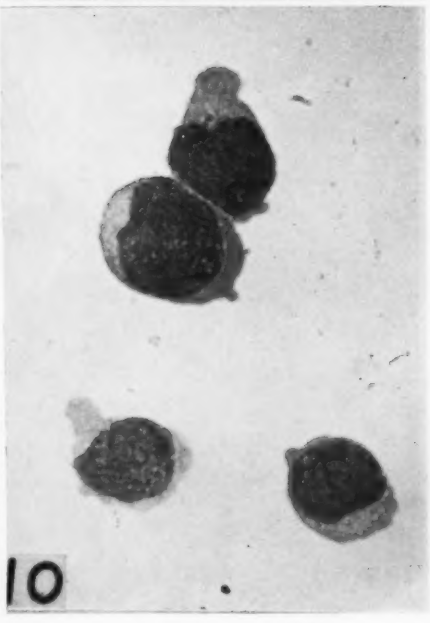
10

Effect of Enol-Ether Derivatives of Dihydrotestosterone on 3-Methylcholanthrene-Induced Rat Mammary Carcinoma [1]

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SUMMARY

A series of enol ethers of dihydrotestosterone, described for the first time and showing low androgenic activity, was tested against hormone-dependent adenocarcinomas that had been induced in Sprague-Dawley rats by oral administration of 3-methylcholanthrene (3-MCA). Two types of tests were conducted: The *prevention test* was designed to determine whether the enol ethers of dihydrotestosterone, given intramuscularly for 50 days, are effective in delaying the average time of the appearance of tumors induced by 10 mg of 3-MCA. This was administered orally and simultaneously with the steroid. The *regression test* evaluated the diminutive effect of the enol ethers, given intramuscularly for 30 days, on mammary cancers induced by 3-MCA. The prevention test showed that most of the enol ethers of dihydrotestosterone efficiently reduced the incidence and delayed the appearance of mammary cancers. In the regression test some of the enol ethers diminished the tumor size to a degree equal to, or even greater than, that observed after ovariectomy or the application of dihydrotestosterone. The regressions were histologically consistent with atrophy of epithelium and sclerosis. The maximal antitumor effect was displayed by dihydrotestosterone enol ethers with an alcohol moiety of 8 or 12 carbon atoms.—J. Nat. Cancer Inst. 27: 1173–1187, 1961.

IN THE past few years we have studied the biological activity of a number of enol ethers derived from steroidal ketones. A characteristic of many Δ^4-3-ketone steroidal enol ethers is their activity, which is greater than that of the parent compounds when given by mouth. Conversely, they are less active if administered parenterally (*1–3*). Sometimes a dissociation of the activities inherent in the parent compound may also be observed. Some biological activities of the latter may remain the same or may even be potentiated in the enol ethers while other effects may have been greatly reduced (*4, 5*). Thus, testosterone and certain of its 17 esters exhibit a high androgenic effect when parenterally administered. This effect is almost completely absent in the respective enol ethers administered by the same route (*6, 7*).

At the suggestion of our laboratory staff a number of testosterone enol ethers have been tried clinically and with success in patients with metas-

[1] Received for publication June 23, 1961.

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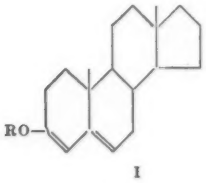
tasizing breast cancer (*8–10*). Sesame-oil preparations were administered intramuscularly. No signs of virilization were observed, which is in agreement with our experiments with animals.

At this point it seemed logical to investigate not only enol ethers of Δ^4-3-ketones (I) but also those of saturated 3-ketones (II), *e.g.*, of 5α-androstan-17β-ol-3-one, which has been recognized experimentally and clinically as one of the most effective inhibitors of mammary cancer.



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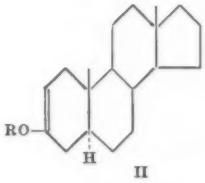


II

R = Alkyl group

The experiments described concern the anticancer effect of enol ethers of dihydrotestosterone on the mammary adenocarcinoma induced in Sprague-Dawley rats by gastric instillation of 3-methylcholanthrene (3-MCA) (*11*). This neoplasm, being hormone-dependent (*11-14*), is to date the only animal tumor that may be used as a model of human breast cancer.

METHODS

Biological.—Female albino rats of the Sprague-Dawley strain, secured from Sprague-Dawley, Inc., Madison, Wisconsin, were kept under standardized conditions. The rats were weaned at the age of 21 days and fed thereafter the synthetic ration 4C of Wissler *et al.* (*15*), which was slightly modified by a supplement of one quarter of an orange for every 4 rats. Drinking water was given *ad libitum*. Ovariectomy was performed under ether anesthesia at the age of 42 days. At the age of 45 days the rats were separated, 8 to 10 to a cage. On the 50th day they were fed, by gastric intubation, 10 mg of 3-MCA in 1 cc of sesame oil 6 days a week for 50 days, according to the method of Huggins *et al.* (*11*). A number of the rats received simultaneous intramuscular injections of 2 mg of the steroid (*prevention test*). After 35 days of treatment, the rats were examined every day for early palpable mammary tumors.

The tumors induced by 3-MCA alone were allowed to grow for 30 days from the date of appearance. These tumor-bearing rats then received intramuscular injections of the steroid for 30 days (*regression test*); biopsies were taken on the preceding day. Every 5 days the growth of the tumor was determined by measurement of its two greatest diameters with a precision caliper. Both bioscopic and necroscopic tissues were fixed in cold acetone for the alkaline-phosphatase reaction (*16*), in Helly's solution for

hematoxylin and eosin, periodic acid-Schiff (PAS), and Alcian Blue stains, and in formalin for Luxol Fast Blue (*17*, *18*).

Chemical.—The steroids were dissolved in sesame oil by gentle heating. The daily dose for each steroid was 2 mg in 0.2 cc of sesame oil. *Throughout this paper dose refers to the amount of steroid administered each day.*

The following code names were used for the compounds under study: 5α-androstan-17β-ol-3-one, androstanolone or dihydrotestosterone (DHT); androstanolone ethyl enol ether (2D-2); androstanolone *n*-amyl enol ether (2Dn-5); androstanolone *n*-heptyl enol ether (2Dn-7); androstanolone *n*-octyl enol ether (2Dn-8); androstanolone *n*-nonyl enol ether (2Dn-9); androstanolone *n*-decyl enol ether (2Dn-10); androstanolone *n*-undecyl enol ether (2Dn-11); androstanolone *n*-dodecyl (lauryl) enol ether (2Dn-12); androstanolone *n*-tetradecyl (myristyl) enol ether (2Dn-14); androstanolone *n*-hexadecyl (cetyl) enol ether (2Dn-16); androstanolone *n*-octadecyl (stearyl) enol ether (2Dn-18).

RESULTS

Prevention Test

The experiments summarized in table 1 were devised to ascertain to what extent dihydrotestosterone enol ethers protect the mammary gland from tumor induction by 3-MCA. The administration of 10 mg 3-MCA by stomach tube 6 days weekly for 50 days induced mammary cancers in all rats, in most cases in less than 60 days. Ovariectomy led to a decrease in tumor incidence and an increase of average time of tumor appearance. When 1 mg testosterone propionate was given intramuscularly concomitantly with the carcinogen, mammary-tumor induction was delayed. The time of tumor appearance ranged from a minimum of 79 to a maximum of 104 days. The concurrent oral administration of 10 mg 3-MCA, and intramuscular administration of 2 mg of dihydrotestosterone proved fatal in all rats before the programmed treatment could be completed. Also several enol ethers of dihydrotestosterone displayed a certain degree of toxicity. This was noticed, however, only with compounds derived from alcohols with the shortest chain or the longest chain of carbon atoms. The intermediate members produced little or no toxicity. Decrease of tumor incidence and of average time of tumor appearance was noted in the surviving rats. Also, gain in body weight was observed in many groups of rats during the treatment.

In this series of experiments 2 mg of 2Dn-8 did not cause any toxic death. In 4 of 6 rats, tumor induction by 3-MCA was delayed as long as 167 days, but not prevented. Rats receiving 2 mg of 2Dn-12 and 3-MCA showed a low mortality and only 1 of 6 exhibited a tumor. It is noteworthy that the neoplasms, which occurred in spite of treatment with 2Dn-8 or 2Dn-12, were fibrotic, as were those observed in ovariectomized rats treated with 3-MCA. Two mg of 2Dn-11 counteracted the

TABLE 1.—Influence of enol ethers of dihydrotestosterone on incidence of palpable mammary tumors in rats treated with 3-MCA.*

Category	Average weight gain (g)	Number of rats	Toxic deaths (during treatment)	Number of rats with tumors	Appearance of palpable tumors (days)		
					Range	Median	Mean
Untreated	81	8	—	—	—		
3-MCA	80	12	—	12/12	43–74	55	57.6 ± 3.3 †
3-MCA + ovariectomy	115	11	4	5/7	53–96	56	70.4 ± 9.5
3-MCA + testosterone propionate, 1 mg	81	8	2	6/6	79–104	100	95.7 ± 4.2
3-MCA + DHT, 2 mg		8	8	—			
3-MCA + 2D-2, 2 mg		7	6	1/1	89		
3-MCA + 2Dn-5, 2 mg	94	7	4	2/3	51–96		
3-MCA + 2Dn-7, 2 mg	134	7	1	2/6	57–99		
3-MCA + 2Dn-8, 2 mg	113	6	—	4/6	93–167		
3-MCA + 2Dn-9, 2 mg	130	8	—	6/8	64–116	68	76.3 ± 8.3
3-MCA + 2Dn-10, 2 mg	141	8	2	6/6	50–102	69	69.7 ± 7.7
3-MCA + 2Dn-11, 2 mg	102	8	4	—			
3-MCA + 2Dn-12, 2 mg	127	8	2	1/6	94		
3-MCA + 2Dn-14, 2 mg	106	6	2	2/4	50–92		
3-MCA + 2Dn-16, 2 mg	142	6	1	3/5	58–67		
3-MCA + 2Dn-18, 2 mg	85	6	4	1/2	68		

*Intact female rats, 50 days of age, were given 10 mg of 3-MCA orally and 2 mg of enol ethers of dihydrotestosterone intramuscularly for 6 days a week for 50 days. The controls received sesame oil. Ovariectomy was performed in 1 group of rats at the age of 42 days. Gain in body weight was recorded for surviving rats at the end of treatment. Rats were observed for 120 days after last day of treatment.

†Standard error.

carcinogenic effect of the polycyclic hydrocarbon, but a high mortality rate was observed.

Regression Test

The tumors induced by oral administration of 3-MCA alone were allowed to grow for 1 month after they appeared. The rats were then given daily intramuscular injections of 2 mg of the steroid under investigation for 1 month. A control group was given 0.2 cc of sesame oil in place of the steroid, and another group of 12 rats was ovariectomized on the 30th day after tumor appearance and received no steroids. The surface area of the tumors was calculated by the product of the 2 greatest diameters of every tumor in each rat. The percent regression (table 2) indicates the diminution of tumors with respect to their initial size. The column showing percent inhibition demonstrates the difference in final tumor size between the treated rats and the untreated controls. The tumors in the control rats receiving injections of sesame oil grew so big that they were double their initial size at the end of the experiment (*see* table 2 and text-fig. 1). There was a remarkable decrease in tumor size in 9 of the ovariectomized rats, while in the other 3 ovariectomy failed to promote any restraint on growth. Two mg of dihydrotestosterone diminished tumor size in all rats. Among the enol ethers of dihydrotestosterone, 2 mg of 2Dn-8 was the most effective agent. Possibly tumor shrinkage was even greater than that caused by dihydrotestosterone. 2Dn-12 (2 mg) showed appreciable antitumor activity (table 2). 2Dn-11, 2Dn-14, 2Dn-16, and 2Dn-18, 2 mg each, promoted a weaker regression than the 8 carbon-alcohol enol ether, 2Dn-8. Also, 2Dn-7, 2Dn-9, and 2Dn-10 had no remarkable activity.

Higher doses, up to 4 mg, of some enol ethers were also tested, but with no more effective results than those obtained with 2 mg. An exception was 2Dn-12, which with a dose of 3 mg. was almost as potent as 2Dn-8 with 2 mg.

Pathology

3-MCA administered by mouth elicited multiple mammary tumors in 100 percent of the rats. The earliest neoplasm was detected by palpation 43 days after the first administration of the carcinogen (table 1). Tumors grew to walnut size within 25 days. Their growth proceeded rapidly thereafter if no treatment intervened. Grossly the tumors appeared soft, fleshy, and vascularized. Their growth infiltrated the adjacent muscles. No metastases were found; severe ulceration and hemorrhages were observed in the animals, which resulted in cachexia.

Histologically, all tumors were adenocarcinomas (fig. 1). They exhibited a variety of glandlike structures lined with many layers of epithelial cells. The lumina were occasionally invaded by papillae. Also they contained secretions which reacted with PAS reagent for neutral mucopolysaccharides and with Alcian Blue for acid mucopolysaccharides. A

TABLE 2.—Influence of hormonal modifications on growth of established mammary tumors in female rats*

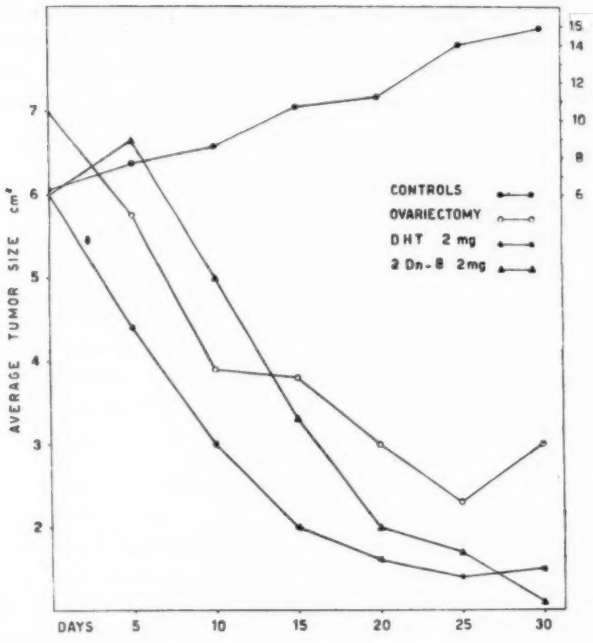
Category	Number of rats showing decreased tumor size, day 30 of treatment	Average initial tumor size cm^2	Average final tumor size, cm^2, day 30 of treatment	Percent tumor regression below initial average size†	Percent tumor inhibition below final control average size‡
Controls	0/12	6. 3	15. 1	—	—
Ovariectomized	9/12	6. 9	2. 9	58. 0	80. 8
Dihydrotestosterone, 2 mg	12/12	6. 0	1. 5	75. 0	90. 1
2Dn-7, 2 mg	9/12	7. 6	4. 5	40. 8	70. 2
2Dn-8, 2 mg	12/12	6. 0	1. 1	81. 7	92. 7
2Dn-9, 2 mg	6/12	7. 5	12. 0	—	20. 5
2Dn-10, 2 mg	7/12	8. 5	10. 2	—	32. 4
2Dn-11, 2 mg	11/12	6. 7	3. 1	53. 7	79. 5
2Dn-12, 2 mg	12/12	8. 6	2. 6	69. 8	82. 8
2Dn-14, 2 mg	10/12	9. 2	7. 6	17. 4	49. 7
2Dn-16, 2 mg	9/10	6. 2	3. 5	43. 5	76. 8
2Dn-18, 2 mg	5/10	6. 3	11. 2	—	25. 8

*Mammary cancer was induced by the intragastric administration of 10 mg 3-MCA 6 days a week for 50 days. The tumors were allowed to grow for 30 days. Then a control group of tumor-bearing rats received intramuscular injections of sesame oil for only 1 month, another group was ovariectomized, and the remaining groups received daily intramuscular injections of steroids for 30 days. The surface area (cm^2) was derived by measuring the greatest diameters of tumors of each rat.

†Percent tumor regression $= \frac{I-F}{I} \times 100$.

‡Percent tumor inhibition $= \frac{U-F}{U} \times 100$.

I = average initial tumor size of treated rats; F = average final tumor size of treated rats; U = average final tumor size of untreated rats.



TEXT-FIGURE 1.—Effects of various treatments on size of 3-MCA-induced tumors. Treatment started 30 days after appearance of tumors. Averages based on total number of rats in each group.

little secretion, presumably composed of phospholipides, stained with Luxol Fast Blue, was sometimes observed in the glands. The stroma contained a PAS-positive substance. Alkaline phosphatase was present in the myoepithelial cells, which were irregularly organized around the neoplastic glands and the luminal border of the epithelium (fig. 2).

As far as the prevention test is concerned, tumors of ovariectomized rats or rats that received 2Dn-7, 2Dn-8, and 2Dn-12 presented quite different features from those of the controls. Macroscopically, they were small and hard. Microscopically, they were composed of many cysts lined by a single row of flat epithelium and embedded in fibrous stroma. No appreciable differences from the controls were noted in tumors of rats treated either with the remaining enol ethers assayed or with testosterone propionate.

In the regression test, biopsies were taken from tumors before they were subjected to enol-ether treatment. The histological examination of necroscopic and bioscopic material from control tumors revealed no differences. Tumors of rats treated for 1 month with 2Dn-7, 2Dn-8, and 2Dn-12, of rats treated with dihydrotestosterone, and of ovariectomized rats, presented a cystic appearance (fig. 3). The cysts were lined by a layer of stretched epithelium and contained a substance positive to PAS

and Alcian Blue. There was no evidence of phospholipides in the test with Luxol Fast Blue. There was alkaline phosphatase in the borders and the interacinal interstices. In some areas where these changes had not yet taken place the epithelium was loaded with fat droplets (fig. 4). Also the interstices among the epithelial cells reacted to alkaline phosphatase.

DISCUSSION

When (10 mg) of 3-MCA was fed to 50-day-old rats 6 days a week for 50 days, multiple mammary cancers usually developed in every animal in less than 60 days (table 1). It was proved that these tumors were hormone-dependent, since they regressed after ovariectomy or hypophysectomy (*11*). Also, their growth was enhanced by critical amounts of estrogens and was abolished by androgens. The hormonal state of the rats was the principal condition responsible for the cancerogenic effect of 3-MCA. When this polycyclic hydrocarbon was fed to ovariectomized rats there was a slowing down in the appearance of cancer. In addition, the tumors grew very indolently and showed sclerosis histologically. When the carcinogen was given together with several enol ethers of dihydrotestosterone, the incidence of tumors was diminished, and the average time of mammary-cancer appearance was remarkably longer than that observed after administration of 3-MCA alone or combined with ovariectomy. Slowly growing tumors appeared very late in a few rats. Testosterone propionate considerably delayed tumor induction by 3-MCA, but did not prevent its growth in any of the animals. Dihydrotestosterone together with 3-MCA caused toxic death of all animals before the programmed treatment was finished. This was an unexpected finding which is now under investigation. However, from the studies of Selye (*19*), it is known that some steroids are not completely devoid of toxicity. It is also known that the oral administration of 3-MCA followed by steroids potentiates the biological activity of the steroids (*20*). Therefore it is likely that in our experiments the 3-MCA enhanced the toxic effect of dihydrotestosterone. The toxicity of dihydrotestosterone appeared greatly reduced in its enol ethers. It was particularly low or even absent in some representatives of this series (2Dn-7, 2Dn-8, 2Dn-9, and 2Dn-16). On the other hand, gain in body weight was observed.

A number of tumors induced by 3-MCA were allowed to grow for 30 days after their appearance and were then subjected to hormonal modifications for 1 month (table 2). Many neoplasms regressed after ovariectomy. Dihydrotestosterone markedly diminished tumor size. Some of its enol ethers equaled or even exceeded the antitumor potency of dihydrotestosterone. 2Dn-8 (2 mg) was the best agent of all the tested steroids. It is interesting to note that in the prevention and regression tests the same two compounds, 2Dn-8 and 2Dn-12, were the most effective

members of the series. The regression of tumors was accompanied by morphologic changes. Ovariectomy, dihydrotestosterone, 2Dn-7, 2Dn-8, and 2Dn-12 diminished tumors to nodules that histologically showed many cysts lined by atrophic epithelium. The cysts contained acid and neutral mucopolysaccharides in larger amounts than found in bioscopic material. There was no appreciable difference in the amounts of these two types of mucopolysaccharides in treated or untreated tumors. Deposits of alkaline phosphatase in the basket cells and the luminal borders of the epithelium were typical of unregressed carcinomas. In cancers with incompletely regressed areas, alkaline phosphatase was also detectable in the epithelial interstices. Once the modifications described had been established, it was not possible to determine which treatment had promoted them.

The administration of more than 2 mg of steroid did not increase proportionally the activity of enol ethers of dihydrotestosterone, and continuing treatment beyond 1 month was of some benefit only in a few instances. All tumors resumed growth about 30 days after the treatment was discontinued, but they often diminished in size when again subjected to treatment.

The mode of action of enol ethers of dihydrotestosterone is not certain. It has been suggested that dihydrotestosterone and other androgenic substances inhibit ovarian and pituitary function (*21*). It appears that the enol ethers also act on experimental mammary cancer through the pituitary, because there is strong evidence, from experiments still in progress, that these compounds are not androgenic while slightly antigonadotrophic. This is true only for the enol ethers of dihydrotestosterone. Those of testosterone are devoid of antigonadotrophic activity.

Throughout the experiments the same daily dose was used for both dihydrotestosterone and its enol ethers; this means that the longer the enol-ether-forming alcohol, the less the proportion of the parent steroid enol, *i.e.*, dihydrotestosterone. Thus, 2 mg of 2Dn-8 and 2Dn-12 corresponded to 1.44 and 1.26 mg, respectively, of free dihydrotestosterone. Nonetheless, these two derivatives proved the best antitumor agents of the enol-ether series, which indicates that the nature of alcohol involved is of great importance in determining the activity of the compounds.

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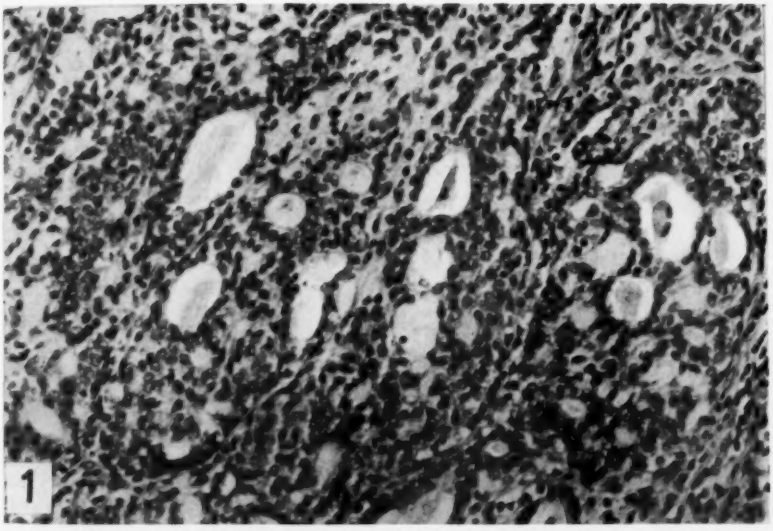
PLATES

PLATE 168

FIGURE 1.—Mammary adenocarcinoma that arose after 3-MCA was fed to an intact female rat. Hematoxylin and eosin. × 180

FIGURE 2.—Distribution of alkaline phosphatase in myoepithelial cells and in luminal border of glandlike structures in mammary cancer induced by 3-MCA. × 300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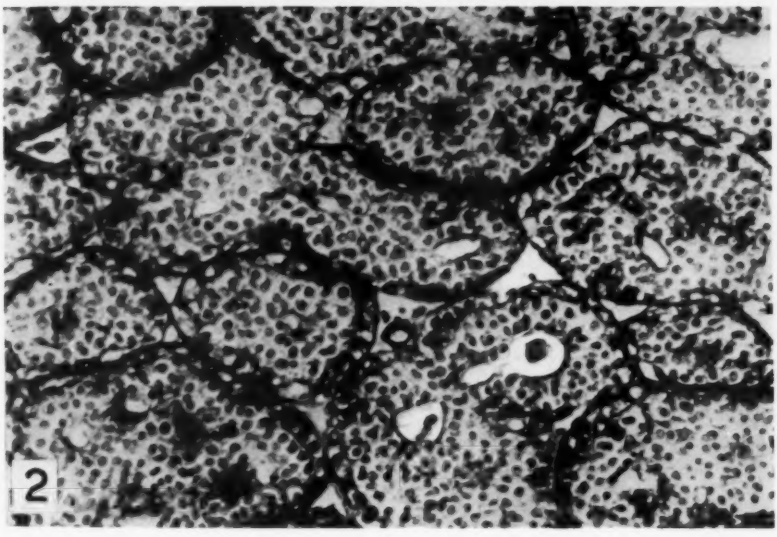
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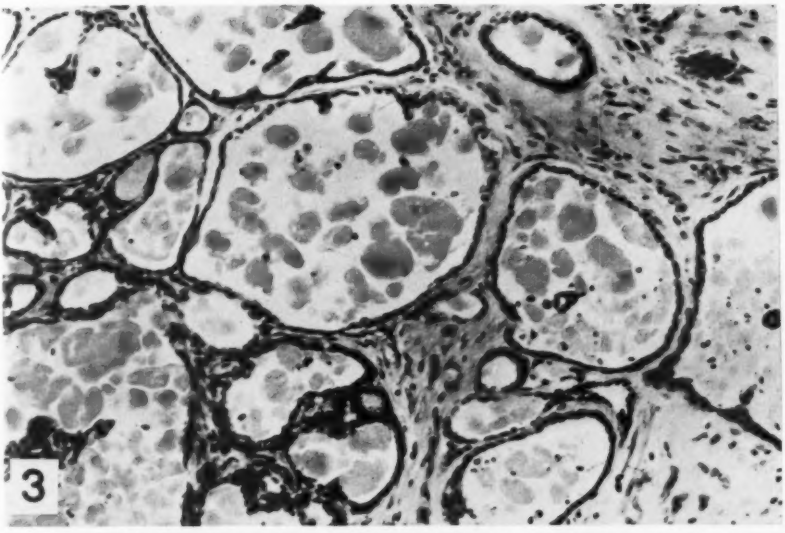
PLATE 169

FIGURE 3.—Cystic appearance of mammary carcinoma after treatment with 2 mg of 2Dn-12 for 30 days. Hematoxylin and eosin. × 140

FIGURE 4.—Lipide-loaded cells in a mammary carcinoma incompletely regressed after 30-day treatment with 2 mg of 2Dn-8. Hematoxylin and eosin. × 180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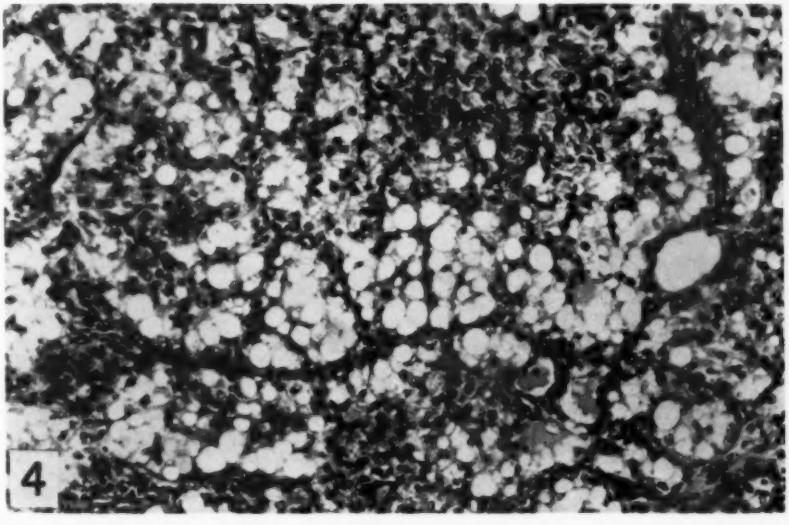
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Elimination of the Effect of the A^y Gene on Pulmonary Tumors in Mice by Alteration of Its Effect on Normal Growth [1]

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SUMMARY

Yellow (A × Y)F_1 mice had more methylcholanthrene-induced pulmonary tumors and greater body weight and body size than did their brown litter mates. This confirmed earlier observations of the effect of the lethal yellow gene A^y on increasing pulmonary tumors and normal growth. When the difference in normal growth due to this gene was reduced by injection of gold-thioglucose, which raised the weight of the browns almost to that of the yellows (whose weight was only slightly increased), the difference in tumor response due to the gene was eliminated. The average number of tumors in the treated browns was raised to a level comparable to that in the treated yellows. Inhibiting the effect of A^y on normal growth by restricting the food intake of the yellows—holding their weight to the level of the browns—reduced the average number of tumors in the yellows to that in the browns. These results suggest that the action of A^y in increasing the occurrence of pulmonary tumors is related to its action in increasing normal growth.—J. Nat. Cancer Inst. 27: 1189–1196, 1961.

FOR SOME TIME we have been interested in the relationship between neoplasia and normal growth and in the effect of specific genes on both. The lethal yellow gene (A^y) of the mouse, first described by Cuénot (*1*), offers an excellent opportunity for study of these relationships. The gene has a lethal effect when homozygous, the embryos dying as blastocysts (*2–4*), but heterozygous animals are viable; their coats are yellow due to restriction of the black pigment of the hair, and their growth rate is greater than that of their nonyellow litter mates. There is some increase in adipose tissue and also an increase in muscular and skeletal growth that is particularly apparent in F_1 hybrids resulting from outcrossing the inbred strain YBR with the gene to other inbred strains (*5–8*). The yellow mice have an increased food intake with reduced energy requirement compared with that of the nonyellows (*9*, *10*).

In (A × Y)F_1 hybrid mice resulting from outcrossing strain A females to strain YBR males it was shown that A^y increased pulmonary tumors when they arose spontaneously or were induced with methylcholanthrene,

[1] Received for publication June 30, 1961.

[2] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

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nitrogen mustard, or urethan, but not when the tumors were induced with dibenz [*a,h*] anthracene (*11–13*). In (C3H × Y)F_1 hybrids resulting from outcrossing strain C3H females to strain YBR males, A^y increased occurrence of hepatomas in the males and increased susceptibility of the females to mammary tumors (*8*). This effect on mammary tumors was expressed as a reduction in tumor age in virgin females. The tumor incidence in both the yellow and the nonyellow virgins approached 100 percent, but the tumors appeared at an average of 8 months in the yellows and at an average of 15 months in the nonyellows. The incidence and average tumor ages in both the yellow and nonyellow breeders were comparable with those of the yellow virgins.

In these previous studies it appeared that the effect of A^y on both the pulmonary tumors and hepatomas may be related to its effect upon normal growth. Furthermore, inasmuch as the effect of A^y on normal growth may be related to endocrine factors, the effect of the gene on mammary tumors may also be closely related. The present paper reports the occurrence of pulmonary tumors in the yellow and the nonyellow (A × Y)F_1 hybrids when we reduced or eliminated the difference in weight due to A^y by raising the weight of the nonyellows with injections of goldthioglucose and by reducing the growth rate of the yellows by restriction of food intake. When the difference in normal growth due to A^y was reduced or eliminated in either manner, the difference in occurrence of pulmonary tumors also was eliminated.

MATERIALS AND METHODS

Strain A females of the $F_{105-107}$ generations of brother and sister inbreeding were mated to yellow strain YBR males of the F_{67-69} brother and sister inbred generations to produce the (A × Y)F_1 hybrids used in this study. Because of the lethal action of A^y when homozygous together with the selection for yellow, these yellow YBR males were heterozygous at this locus (A^ya) but, since they were highly inbred, they could be assumed to be homozygous at practically all other loci. The strain A mice are *aa* and, since they are highly inbred, can also be expected to be homozygous at all other loci. These (A × Y)F_1 hybrids, then, present an isogenic system in that (aside from sex-chromosome differences) they all would be genetically alike except for segregation at the A^y locus. Half of them would be expected to be yellow A^ya and half nonyellow *aa*, which is brown in this case, since both parent strains carry brown. Any difference between the two color groups when exposed to the same environment or treatment can, therefore, be attributed to the A^y gene.

The F_1 hybrids were weaned at 4 weeks of age. They were individually identified and the coat color of each was recorded. They were then segregated as to sex, but not as to color, and throughout the study were kept in plastic cages with 8 mice to the cage except for those in the food-restriction experiment. All animals were fed Purina laboratory chow,

given an unlimited supply of tap water, and housed in an air-conditioned room at 78° F.

The mice were divided into groups for three experiments, with the sexes and genotypes distributed in each group as shown in table 1. The first experiment was to measure again the effect of A^y on the number of pulmonary tumors induced with methylcholanthrene; the second, to ascertain whether reduction of the weight difference by the injection of both color groups with goldthioglucose would eliminate the effect of A^y on the number of induced tumors; and the third, to ascertain whether holding down the weight of the yellow mice by restriction of food would eliminate the effect of the gene on the occurrence of the tumors.

At 8 weeks of age all animals of the first experiment were weighed and each received an injection intravenously of .25 mg of methylcholanthrene dispersed in .25 cc of distilled water. At 5 weeks of age all the yellow and brown mice of the second experiment received an injection intraperitoneally of .2 cc of a 10 percent solution of goldthioglucose. Following the usual procedure of Brecher, Waxler, and coworkers (*14*, *15*), to insure survival, we removed the food from the animals on the day before the injection and returned it on the day after. Thereafter their weights were recorded at weekly intervals. Then at 8 weeks of age, when the animals were beginning to show the obese effects of the goldthioglucose, they received the same injection of methylcholanthrene as those of the first experiment.

In the third experiment, pairs of yellow and brown litter mates were set aside at weaning time, and thereafter each yellow mouse was allowed only enough food to maintain its weight equal to or slightly less than that of its brown mate, and weights and food intake were recorded daily. Later, at 8 weeks of age, all mice of the third experiment received an injection of methylcholanthrene identical to that in the first and second experiments.[3]

All animals of the three experiments were weighed finally, killed, and examined at 20 weeks of age, 12 weeks after the injection of the methylcholanthrene. Before the lungs were removed from the chest cavity approximately 1 cc of Tellyesniczky's fluid (70% ethyl alcohol, 20 parts; formalin, one part; glacial acetic acid, one part) was injected intratracheally to distend the lungs and make the tumors stand out more clearly. The lungs were then removed and the tumors appearing on the surface were counted with the aid of the dissecting microscope. The total body length of each animal from the tip of the nose to the tip of the tail was measured, the left gastrocnemius muscle of each was dissected

[3] The dose of methylcholanthrene was not on a per body-weight basis, but all animals received .25 mg. All animals were weighed, however, at the time of injection of the methylcholanthrene and their average weights were as follows: experiment 1, $A^y a$ males 30.9 g, aa males 27.0 g, $A^y a$ females 25.7 g, and aa females 21.0 g; experiment 2 in which the animals received goldthioglucose, $A^y a$ males 29.9 g, aa males 28.0 g, $A^y a$ females 27.9 g, and aa females 25.4 g; and experiment 3 in which the food of the yellow mice was restricted, $A^y a$ males 24.2 g, aa males 26.5 g, $A^y a$ females 20.3 g, and aa females 21.2 g. A uniform dose of .2 cc of a 10 percent solution of goldthioglucose was also given to all animals of experiment 2. At the time they received the goldthioglucose their average weights were as follows: $A^y a$ males 17.6 g, aa males 16.9 g, $A^y a$ females 15.6 g, and aa females 14.4 g.

TABLE 1.—Pulmonary tumors and body measurements in (A × Y)F_1 mice receiving injections of methylcholanthrene at 8 weeks of age and autopsied at 20 weeks

Experiment	Number of mice	Genotype	Sex	Treatment	Incidence of tumors (percent)	Mean No. of tumors	Mean body weight (g)	Mean body length (cm)	Mean weight, left gastrocnemius muscle (g)	Mean length, left femur (cm)
1	47	*A^{v}a*	♂	None	97. 9	7. 7	48. 5	20. 7	0. 204	1. 60
	53	*aa*	♂	None	92. 5	4. 2	35. 2	20. 1	0. 201	1. 59
	46	*A^{v}a*	♀	None	100. 0	13. 9	45. 6	20. 0	0. 162	1. 59
	63	*aa*	♀	None	98. 4	7. 4	26. 0	19. 3	0. 147	1. 56
2	59	*A^{v}a*	♂	Goldthioglucose	100. 0	9. 0	50. 8	19. 9	0. 176	1. 55
	76	*aa*	♂	Goldthioglucose	98. 7	9. 9	45. 4	19. 6	0. 174	1. 54
	60	*A^{v}a*	♀	Goldthioglucose	100. 0	12. 5	50. 5	19. 5	0. 155	1. 55
	82	*aa*	♀	Goldthioglucose	100. 0	12. 6	42. 7	19. 4	0. 149	1. 55
3	71	*A^{v}a*	♂	Food restricted	91. 5	4. 4	32. 1	20. 3	0. 168	1. 57
	73	*aa*	♂	None	95. 9	4. 8	34. 4	20. 4	0. 192	1. 60
	52	*A^{v}a*	♀	Food restricted	100. 0	6. 2	26. 1	19. 8	0. 131	1. 56
	52	*aa*	♀	None	98. 1	6. 4	26. 1	19. 7	0. 142	1. 58

out and weighed, and the left femur of each was dissected out and measured in length.

RESULTS AND DISCUSSION

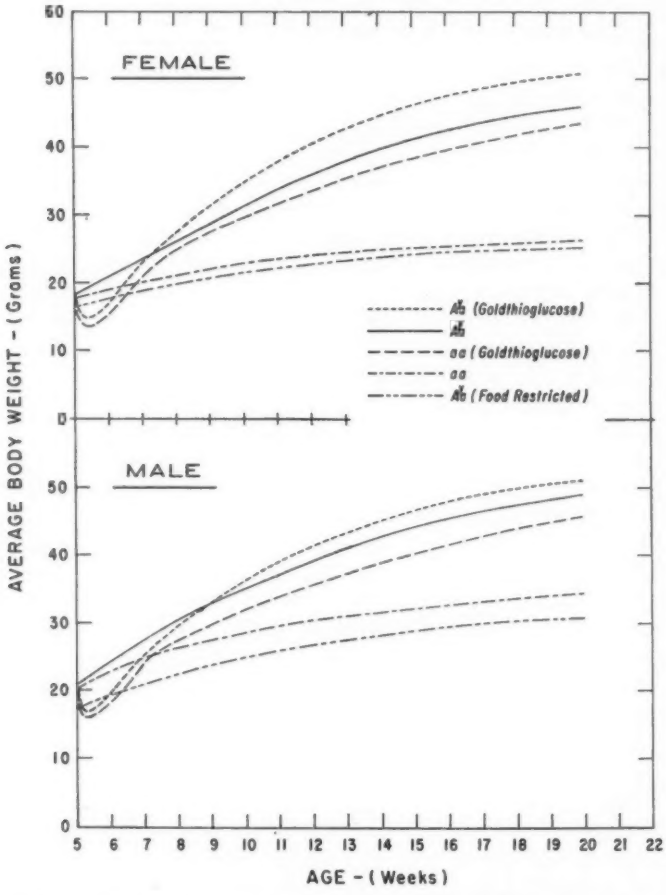
The results of the three experiments are recorded in table 1. Pulmonary tumors were the only neoplasms that occurred in the animals of these relatively short-term studies. All or nearly all animals in each group had at least one pulmonary tumor, so differences in tumor response were noted in number of pulmonary tumors in each mouse. Since in all experiments the females gave a higher tumor response than the males, and since there were also sex differences in body weights and measurements, the data for the sexes are presented separately.

The results from experiment 1 confirmed our earlier observation that A^y increases the occurrence of pulmonary tumors induced with methylcholanthrene. The average of 7.7 tumors in the yellow males was significantly greater than the average of 4.2 tumors in the brown males ($t = 4.45$; $P<0.01$), and the average of 13.9 tumors in the yellow females was significantly greater than the average of 7.4 in the brown females ($t = 6.62$; $P<0.01$).

The effect of A^y on body size of animals that received only methylcholanthrene can also be noted in the data of this first experiment. The yellow males were significantly heavier than the brown males ($t = 29.67$; $P<0.01$), and the yellow females were significantly heavier than the brown females ($t = 33.40$; $P<0.01$). Body length from the tip of the nose to the tip of the tail was significantly greater for the yellow males than for the brown males ($t = 7.46$; $P<0.01$), and significantly greater for the yellow females than for the brown females ($t = 6.62$; $P<0.01$). The mean weight of the left gastrocnemius muscle of the yellow males was greater than that of the brown males, but not significantly greater. However, the mean weight of the left gastrocnemius muscle of the yellow females was significantly greater than that of the brown females ($t = 8.87$; $P<0.01$). The mean length of the left femur of the yellow males was slightly greater than that of the brown males but not significantly greater, while the mean length of the left femur of the yellow females was significantly greater than that of the brown females ($t = 5.10$; $P<0.01$).

As had been noted by others (*14*, *15*), weight response to the goldthioglucose in experiment 2 was somewhat irregular. The weight of a few animals did not even exceed the average weight of those of experiment 1 that did not receive the goldthioglucose. All animals that received goldthioglucose, however, were included in the calculation of the mean tumor response, weights, and measurements recorded in the table. The goldthioglucose resulted in an increase in mean body weight of both color groups of both sexes (text-fig. 1 and table 1), but the increase was greater in the brown animals of both sexes than in the yellow animals of both sexes, thus reducing the effect of A^y on body weight. It should

be noted, however, that the goldthioglucose did not increase body length, length of femur, or weight of the gastrocnemius muscle. In fact, most of these measurements were even less than those of the corresponding groups without the goldthioglucose. Thus, the increase in body weight of these treated animals represented an increase in adipose tissue.



TEXT-FIGURE 1.—Growth curves of (A × Y)F_1 male and female mice.

The principal point to be noted in experiment 2 is that the injection of goldthioglucose with the resulting rise in body weight, particularly of the brown animals, raised the tumor response of the brown animals to a level almost identical with that of the yellows, as can be seen in the table. Thus, when the effect of A^y on body weight was reduced in this manner, its effect on occurrence of pulmonary tumors was eliminated.

In experiment 3, the weights of the yellow mice were successfully held

to a level equal to or below that of the browns by careful restriction of food (text-fig. 1 and table 1). In line with Dickerson and Gowen's (*10*) previous finding that normally yellow mice not only have a higher food intake than their nonyellow siblings but also have a more efficient utilization of food, it was found that to hold the weight of the yellow mice down to slightly below the level of that of the browns, their food intake had to be considerably below that of the browns. The brown males that were permitted to eat all they wanted ate, on the average, 3.8 g per day, whereas the food intake for the yellow males was only 3.1 g per day. The brown females ate an average of 3.4 g of food per day, whereas the yellow females had to be limited to an average of only 2.8 g per day to maintain their weight at almost exactly that of the browns.

In holding the weight of the yellow mice to that of the browns we found that skeletal growth as indicated by both total-body length, and length of femur was likewise held to approximately that of the browns. Muscle weight in the restricted yellows, however, was held considerably below that of the browns.

By inhibiting the effect of the A^y gene on body weight through restriction of food intake, we also inhibited its effect on tumor response. The average number of tumors in the brown males and females of experiment 3 was approximately that of the brown males and females of experiment 1, as would have been expected. The average number of tumors in the food-restricted yellow males and females of experiment 3 was not as high as that in the nonrestricted yellow males and females of experiment 1, but was almost exactly the same as that in the brown mice with which they had been paired.

These results indicate that the action of the lethal yellow gene A^y on increasing normal growth is related to its action on increasing occurrence of pulmonary tumors. What then is the more nearly primary action of A^y and is there one such action resulting in all the observed phenotypic effects? If there is a single basic action, the fact that the gene restricts the melanin pigment of the hair and leaves the yellow pigment suggests that this action may involve one of the enzymes in the metabolism of tyrosine. Could the gene, when homozygous, cause some deficiency here that has a lethal effect in the early embryo? Could such action of the gene, when heterozygous, result in the increased normal growth and at the same time the increased occurrence of pulmonary tumors, both spontaneous and induced? Could such metabolic action also increase the occurrence of hepatomas as noted in (C3H $\times$ Y)F_1 males where not only A^y increased hepatomas but where there was a positive correlation between normal growth and occurrence of hepatomas? The effect of A^y on mammary tumors appears to be related to endocrine factors. Could the suggested effect of A^y on some step in the metabolism of tyrosine result in endocrine alteration which in turn affects the occurrence of mammary tumors or even results in the observed changes in growth? With the development of biochemical genetics in the mouse it is hoped that such questions may eventually be answered.

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Simple and Radical Mastectomy for Breast Cancer: A Re-Analysis of Smith and Meyer's Report From Rockford, Illinois [1]

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SUMMARY

Analysis of 448 women treated surgically for cancer of the breast in Rockford, Illinois, between 1929 and 1954 indicated that the 5- and 10-year survival rates are the same whether radical or simple mastectomy was performed. There are detectable differences in the selection of patients for different operations, however, so that as in other retrospective analyses, exact comparability between the groups cannot be demonstrated. A rigidly controlled clinical trial, with patients entering various surgical procedures to be compared by randomization, is needed and urged.—J. Nat. Cancer Inst. 27: 1197–1215, 1961.

SMITH AND MEYER (*1*) reported in 1959 the survival rates of 448 patients with breast cancer treated in Rockford, Illinois, between 1924 and 1952. Of 432 patients followed for at least 5 years, 324 patients underwent an operation classified as radical mastectomy; of these, 173, or 53 percent, were alive at 5 years; of 97 patients whose operation was classified as simple mastectomy, 52, or 54 percent, were alive at 5 years; and of 11 patients who had local excision of the tumor, 6 were alive 5 years later. The 10-year survival rates for radical and simple mastectomy were 30 and 32 percent, respectively.

Smith and Meyer concluded that the survival rates were not affected by the type of operation performed. This conclusion is valid to the degree that it can be demonstrated that the groups are comparable with respect to the disease and the patients, and to the degree that selection factors can be adjusted.

It is obvious that the controversies between the proponents of the radical mastectomy and those who espouse either less or more extensive resections for mammary carcinoma can be resolved only through carefully designed clinical trials in which patients would enter various treatment groups by a randomization scheme, which would thus minimize known and unknown factors of selection. In the continued absence of such studies, data from a single community or center in which several therapeutic modalities have been followed may represent a better source for valid

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comparisons than data from widely separated areas where unknown variables are even more likely to be introduced.

An independent re-analysis of the data from Rockford was therefore deemed important. The Biometry Branch of the National Cancer Institute was provided this opportunity during the summer of 1960. The original hospital records were examined, and all abstracts and analyses were prepared independently of the original investigators. We are greatly indebted to Drs. Smith and Meyer for their cooperation in permitting us to carry out this re-analysis.

MATERIAL

Records on all patients with breast cancer that were surgically treated in Rockford hospitals were examined, abstracted, and the data transcribed on punch cards.

The period chosen for analysis was 1929 through 1954, instead of Smith and Meyer's 1924–52 period. Two additional years of 5-year follow-up had become available and made it possible to replace the less complete data from 1924–28 with more reliable recent data. The total number of surgically treated cases was 465, approximately the same number as Smith and Meyer's 448 patients.

Of the total 465 cases, 17 were excluded from the main analyses for the following reasons:

Three were males. Two were treated by radical mastectomy and died 15 and 32 months later. One had a simple mastectomy and is alive at 6 years.

Four women had simple mastectomy performed only as part of palliative management of disseminated disease with clinically demonstrated visceral or skeletal metastasis. All died within 13 months of surgery. These cases were considered not to have been eligible for definitive surgical treatment for breast cancer.

Ten women were recorded as having had only local excision of the breast cancer. These patients had excisional biopsies that were not followed by further surgery due to the choice of the patient or the physician. Five were alive at 5 years; of the 8 followed for 10 years, 2 were alive. This was obviously an unusual group; 5 of the women were over 70 years old, and the average greatest diameter of the excised tumors was only 2.7 cm.

Of the total 17 excluded cases, therefore, 6 were alive at 5 years. The exclusion of these cases in the subsequent analyses, in which the chief aim is to compare the results of radical mastectomy with simple mastectomy, does not invalidate any major conclusions that are reached.

There were 6 postoperative deaths. Two occurred during the 1929–41 period after radical mastectomy. One occurred during the 1942–47 period after a modified radical operation. All 3 deaths after 1948 were in patients subjected to simple mastectomy. These 6 cases are included in all subsequent analyses.

DEFINITIONS

The 448 women with surgically treated breast cancer were categorized on the basis of the following definitions, similar to those used in an earlier study (*2*):

1) *Diagnosis.*—Positive pathologic diagnosis of carcinoma of the breast, based on the histologic examination of the primary lesion, was available for all patients.

2) *Surgery.*—The cases were divided into three groups: radical, modified radical, and simple mastectomy. A radical mastectomy required an adequate description of the operation, which indicated dissection of the axilla, both pectoral muscles and the breast with generous skin margins, and confirmatory evidence of the presence of these tissues in the surgical specimen. If the operation was listed as a radical mastectomy in the operation report, but the records could not substantiate the full requirements as previously stated, the patients were classified as having had a modified radical mastectomy. Simple mastectomy usually consisted of removal of the breast only. However, in some cases, some of the axillary contents or a portion of pectoralis major muscle was also removed, but not the pectoralis minor. Thus, information on some lymph nodes from the axilla was available in 50 of the 103 simple mastectomies.

3) *Stage of disease.*—Patients who underwent radical mastectomy were classified as having localized disease if the axillary lymph nodes were histologically free of tumor, and if the primary tumor was not ulcerated, not fixed to the chest wall, and had no satellite nodules. The patients were classified as having regional involvement if the lymph nodes contained tumor, or the primary lesion was ulcerated, fixed, or had satellite nodules.

In the majority of patients who underwent simple mastectomy and several who had a modified radical mastectomy, the pathologic information was insufficient for adequate or comparable staging, since lymph nodes were either not available or represented a smaller sample than among the radical resections. If the primary tumor was not ulcerated, fixed to the chest wall, or had satellite nodules, and if there was no evidence of axillary involvement, the cases were classified as localized. If the primary tumor was ulcerated, fixed, or had satellite nodules, and if the histologic examination of lymph nodes showed tumor, the cases were classified as regional. When these data were neither available nor interpretable, the cases were classified as being of an indeterminate stage.

4) *Size of tumor.*—Since a relationship between the size of the primary tumor and prognosis has been reported (*3*), an analysis was made of the size of the primary tumor as measured or estimated from the surgical specimen. For this purpose, tumors less than 3 cm in the greatest diameter were designated as small, those between 3 and 6 cm, as medium, and over 6 cm, as large.

5) *Follow-up.*—Since the period chosen for analysis was 1929 through 1954, all patients were eligible for at least 5 years of follow-up. Patients

lost to follow-up are defined as any patients not recorded as dead who were followed less than 10 years or, for those who had surgery in 1950 or later, were not followed at least into 1960. Of the 448 patients, 15 were lost to follow-up, 11 during the first year after surgery, and one each during the 3d, 5th, 6th, and 9th years. Thus, the over-all follow-up was 97 percent.

6) *Cause of death.*—No attempt was made to determine the presumed cause of death. All patients not recorded as dead were known to be alive in 1960 (except for 15 with unknown survival status), and all dead and lost patients were included in the analyses. All deaths within 30 days after mastectomy were classified as postoperative deaths and are also included in the analyses.

STATISTICAL METHODS

The life-table or actuarial method as described by Cutler and Ederer (*4*) was used for the computation of observed survival rates. Using the calculated observed rates and expected rates estimated from population life tables, we computed the relative survival rates with the methods described by Cutler *et al.* (*5*).

The relative survival rate is defined as the ratio of the observed survival rate in a group of patients, during a specified time interval, to the expected rate. The expected survival rate is that for a group similar to the patient group in such variables as age, sex, and race, but free of the disease in question.

For most of the present analyses, United States Abridged Life Tables for 1945 were used in the estimation of expected survival rates. However, when the data were subdivided into 3 time periods, *i.e.*, prewar, war, and postwar years, expected rates were estimated with 1940, 1945, and 1950 United States life tables, respectively.

Standard errors for the relative survival rates were computed to indicate to what extent the survival rates may have been influenced by sampling variation (*6*). For example, by adding and subtracting twice the standard error to and from the relative survival rate, one obtains an approximate 95 percent confidence interval. This means that in repeated observations under the same conditions the true survival rate will lie within a range of two standard errors on either side of the computed rate, an average of 95 times in 100.

ROCKFORD

Rockford is the second largest city in Illinois, straddling the Rock River in the northwest corner of the State some 100 miles from Chicago. It is in a rich agricultural area contiguous with Wisconsin and is an industrial city with machine tool, automotive parts, furniture, and screw manu-

facture among the important items of its light-to-medium industry. The population, almost exclusively white, with about one third of Scandinavian descent, is now 126,000, having grown from about 80,000 during the past 25 years. During the war years, there was a depletion of the young adult population due to the draft and migration to war industries.

Until the postwar period, surgery in Rockford was generally performed by physicians who did not limit their practice to surgery, and referral to Chicago or Rochester, Minnesota, was more common than now. During the war, Rockford experienced a shortage of physicians, particularly surgeons, due to the demands of the armed forces. After the war, the city attracted surgical specialists with extensive formal training, and there are now some 25 men who limit themselves to surgery. Operations are performed in the three community hospitals, Rockford Memorial, Swedish-American, and St. Anthony's, with a present capacity of about 800 beds.

The changes that occurred in the population of Rockford and its physicians are reflected in the data on surgically treated breast cancer. When the 448 patients were tabulated by individual years of operation and type of operation, it was obvious that most of the simple mastectomies were performed during the war years, 1942–47. This, in effect, produced a clinical experiment in which the results during the war period could be compared with the periods before and after the war. The analyses were therefore oriented to a comparison of results during 3 periods: prewar (1929–41), war (1942–47), and postwar (1948–54). It seemed reasonable to postulate that if radical mastectomy yields better results than simple resection, survival among patients during the pre- and postwar periods would be better than during the war period. In addition, the postwar period is exemplified by better trained surgeons, emphasis upon earlier recognition and more aggressive treatment for cancer, and the introduction of hormone management of patients with recurrent disease.

TOTAL DATA

Table 1 presents the distribution of patients by type of operation and by the 3 time periods. The proportion of patients treated by simple

TABLE 1.—Distribution by type of operation and time period: patients with cancer of the breast treated surgically in Rockford, Illinois, 1929–54

Mastectomy	1929–41		1942–47		1948–54		Total	
	Number	Percent	Number	Percent	Number	Percent	Number	Percent
Radical	49	44	40	31	148	72	237	53
Modified radical	45	40	36	27	27	13	108	24
Simple	18	16	55	42	30	15	103	23
Total	112	100	131	100	205	100	448	100

TABLE 2.—Survival rates by time period of operation: patients with cancer of the breast treated surgically in Rockford, Illinois, 1929–54

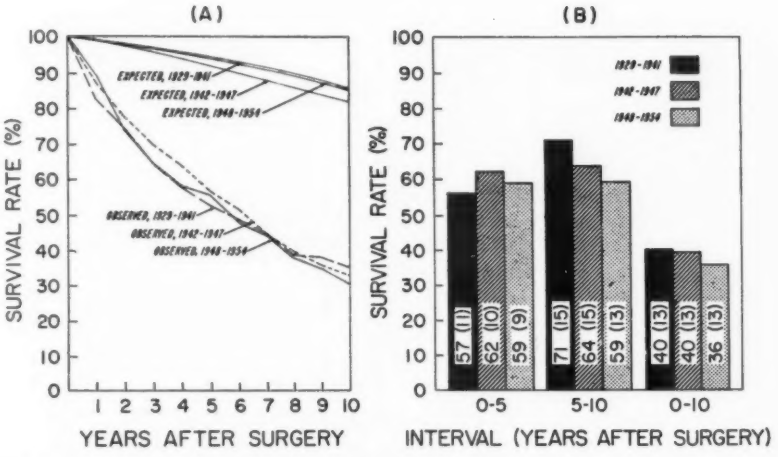
Time period	Total No. of cases	Years after surgery	Died during interval	Lost to follow-up during interval	Withdrawn alive during interval	Effective sample size*	Percent surviving interval			2 SE of relative rate†
							Observed	Expected	Relative	
1929–41	112	5	50	7	0	106	53	94	57	11
		10	69	7	0	105	35	86	40	13
1942–47	131	5	55	2	0	129	57	92	62	10
		10	87	2	0	129	33	82	40	13
1948–54	205	5	90	3	0	202	56	94	59	9
		10	125	6	56	138	31	86	36	13

*The equivalent sample size for a cohort in which there are no losses to follow-up or withdrawals alive, but which yields the same observed survival rate with the same standard error (4).
† Two standard errors of the relative survival rate.

mastectomy is substantially higher during 1942–47 than during the earlier or later periods.

Table 2 is a summary showing the survival rates, at 5 and 10 years after surgery, obtained from a year-by-year life-table analysis. This table shows that the survival of the patients is practically the same for the 3 time periods, despite the greater proportion of simple mastectomies during the war period. The slightly lower expected rate during the 1942–47 period is due to the older age distribution of patients, which is dealt with separately later. However, the conclusion is the same whether the observed or the relative survival rates are used; all differences are well within 2 standard errors, or not statistically significant.

These data, by annual survival over a 10-year period, are presented graphically in text-figure 1. The survival curves for the 3 time periods are very similar.



TEXT-FIGURE 1.—(A) Observed and expected cumulative survival curves by time period of operation. (B) Relative survival rates for specified follow-up intervals by time period of operation: patients with cancer of the breast in Rockford, Illinois, 1929–54. Two standard errors of the relative survival rate given in parentheses.

Also shown in graphical form are the relative survival rates by time period of operation. With the use of the observed and expected rates experienced by the 5-year survivors, the additional relative rate for the 5–10-year interval was also computed. It is of interest that the survival rates in the second 5-year interval are only slightly better than for those in the first 5 years after surgery.

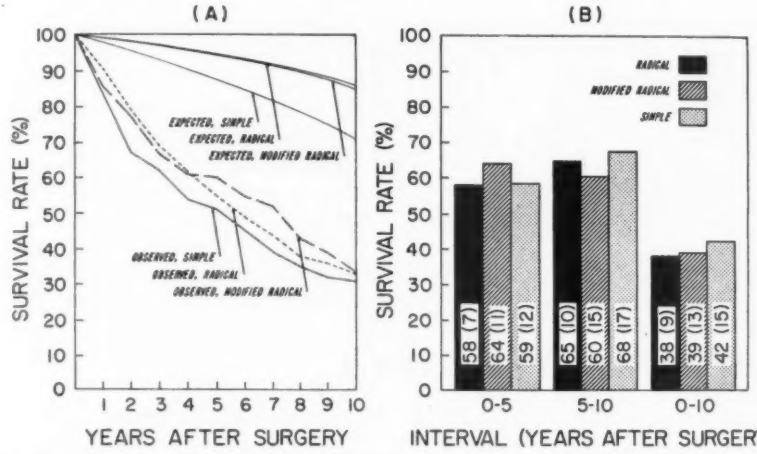
Table 3 analyzes survival when the data are arranged by type of operation, independent of the time periods. Whether the observed or the relative rates are considered, survival is similar for the 3 types of operation.

TABLE 3.—Survival rates by type of operation: patients with cancer of the breast treated surgically in Rockford, Illinois, 1929–54

Type of operation	Total No. of cases	Years after surgery	Died during interval	Lost to follow-up during interval	Withdrawn alive during interval	Effective sample size*	Percent surviving interval			2 SE of relative rate†
							Observed	Expected	Relative	
Radical	237	5	103	9	0	229	55	95	58	7
		10	142	10	49	185	33	87	38	9
Modified radical	108	5	42	3	0	106	60	94	64	11
		10	68	4	4	100	34	87	39	13
Simple	103	5	50	0	0	103	51	88	59	12
		10	71	1	3	101	31	72	42	15

*The equivalent sample size for a cohort in which there are no losses to follow-up or withdrawals alive, but which yields the same observed survival rate with the same standard error (4).
† Two standard errors of the relative survival rate.

The data by operation are presented graphically in text-figure 2. The slopes of the 3 curves are similar, and the slightly higher mortality experienced by simple mastectomy is compensated by the higher expected mortality for this older age group.



TEXT-FIGURE 2.—(A) Observed and expected cumulative survival curves by type of operation. (B) Relative survival rates for specified follow-up intervals by type of operation: patients with cancer of the breast in Rockford, Illinois, 1929–54. Two standard errors of the relative survival rate given in parentheses.

The graphical presentation of the relative survival rates points out that any differences observed during the first 5-year period are practically reversed during the second 5 years, which results in almost identical 10-year rates.

The conclusion perforce is that no superiority in survival is demonstrable among women treated by radical mastectomy as compared with the results after simple mastectomy.

MODIFYING FACTORS

Analyses were carried out on the possible role of various factors that may either strengthen the acceptability of the groups as being comparable, or reveal sources of selection. In the latter instance, the findings were tested for the direction and degree such factors might contribute to differences in survival rates.

Stage of Disease

Table 4 shows the stage distribution of the patients in the 3 operation procedures.

TABLE 4.—Analysis by stage of disease: patients with cancer of the breast in Rockford, Illinois, 1929–54

Stage	Type of operation						Total		Relative survival rates*	
	Radical		Modified radical		Simple					
	Number	Percent	Number	Percent	Number	Percent	Number	Percent	5-year	10-year
Localized	98	41	48	44	22	21	168	38	81(8)	58(11)
Regional	139	59	55	51	39	38	233	52	43(7)	22(8)
Indeterminate	0	0	5	5	42	41	47	10	67(17)	57(23)
Total	237	100	108	100	103	100	448	100	60(5)	39(6)

* Two standard errors of the relative rate in parentheses.

Since 53 of the simple mastectomies consisted of removal of the breast without the axillary contents, and in most others the axillary dissection was incomplete, it was impossible to determine the stage of the disease for this operation group on the same basis as for patients with radical mastectomy. The large proportion of cases classified as indeterminate stage among patients treated by simple mastectomy makes difficult any meaningful direct comparisons of end results cross-classified by stage and type of operation. Nevertheless, since the stage of the disease is the most important single determinant of prognosis in breast cancer, it seemed useful to examine the trends in the results during the 3 time periods by stage of the disease and type of operation.

Table 5 summarizes these analyses. Despite the small number of cases in most cells, some possible effects and their tentative interpretations are suggested.

The stage distributions by time periods as shown in table 5 indicate that there was an increase in the proportion of localized cases during the last time period. Even if all cases with indeterminate stage were localized, the percentages of localized cases for the 3 successive time periods would be 42, 43, and 54, respectively.

The stage distributions and survival data in Rockford were compared with the combined experience of 7 large hospital centers in the United States, as reported at the Fourth National Cancer Conference (7). The ratios of known localized to known regional cases are very similar, and both sources indicate a shift after 1950 toward a greater proportion of localized cases. The survival rates by stage of disease are also practically identical for both series. Thus, the experience at Rockford seems similar to that elsewhere in the United States.

The important point, however, is that the shift toward a greater proportion of cases classified as being localized during the most recent period in Rockford was not reflected by better survival rates. Also, there is no evidence that the second time period, when many patients were treated by simple mastectomy, was characterized by a greater proportion of localized cases.

Inspection of the relative survival rates for the 3 types of operation during the 3 time periods, as given in table 5, leads to several additional interesting interpretations.

The relative survival rates during the first time period, 1929–41, were higher for patients treated by simple mastectomy than for those treated by radical mastectomy. The results obtained with radical mastectomy during this calendar period were particularly poor. Of the 49 patients treated by radical mastectomy, 11 died within 1 year of operation. Two of these deaths were postoperative. In contrast, only 2 of the 40 patients undergoing radical mastectomy in the period 1942–47 died during the 1st year.

During the second time period, 1942–47, the survival rates for the 3 operations were very similar. If there was any difference, it was in

TABLE 5.—Analysis by time period and type of operation: patients with cancer of the breast in Rockford, Illinois, 1929–54

Time period	Type of operation	Total No. of patients	Stage distribution (%)			Relative survival rates*	
			Localized	Regional	Indeterminate	5-Year	10-Year
1929–41	Radical	49	24	76	0	40(16)	28(18)
	Modified radical	45	44	49	7	67(16)	43(20)
	Simple	18	22	33	45	73(26)	68(34)
	Total	112	32	58	10	57(11)	40(13)
1942–47	Radical	40	28	72	0	58(19)	36(22)
	Modified radical	36	36	58	6	66(20)	40(25)
	Simple	55	14	44	42	64(17)	44(23)
	Total	131	24	57	19	62(10)	40(13)
1948–54	Radical	148	51	49	0	63(10)	42(16)
	Modified radical	27	56	44	0	58(24)	32(36)
	Simple	30	33	30	37	40(25)	8(25)
	Total	205	49	46	5	59(9)	36(13)

* Two standard errors of the relative rate in parentheses.

favor of the simple mastectomy, but the large standard errors preclude any firm conclusions.

During the third time period, 1948–54, the survival rates among patients that underwent simple mastectomy are lower than the survival rates after radical mastectomy. This is an excellent manifestation of selection; by this time, the simple mastectomy was reserved for older women with contraindications for more extensive surgery. It is of note that during this period the 3 postoperative deaths were encountered after simple mastectomy. Such selection in favor of the radical mastectomy, however, does not result in an increased total survival rate during this time period.

Comparison of stage distribution can be made on firmer grounds between the radical and the modified radical mastectomy groups than between these two groups and the simple mastectomy cases. Table 5 shows the 5-year survival rates after radical and modified radical mastectomy for the 3 time periods. In the first period, the large difference in survival in favor of the modified radical is statistically significant. Correspondingly, the percentage of localized cases in the modified radical groups is much higher than in the radical group. However, in the 2 later periods the survival rates for these 2 groups are not significantly different and the percentages of localized cases are also similar. Thus, the larger proportion of localized cases in the modified radical mastectomy group probably accounts for its better survival rates in the early time period.

Size of Tumor

Table 6 shows the distribution of the size of the primary breast cancer, as measured or estimated in the surgical specimen, according to the type of operation. The distributions are not significantly different among the 3 operation groups, though patients classified as modified radical have somewhat higher percentages of medium and large tumors.

Survival of patients when classified against size of the tumor reveals an inverse association. The difference in survival between the small tumors and the large ones is statistically significant. There is also an association between stage and tumor size: The proportion of small tumors was higher among patients having localized disease than among patients with regional involvement. The proportion of small tumors increased during more recent time periods: During 1929–41, 38 percent of the tumors were small; during 1942–47, 47 percent; and during 1948–54, 49 percent.

Thus, the patients that underwent simple mastectomy are not demonstrated to have had a higher proportion of smaller tumors with better prognosis than was found in patients receiving radical mastectomy.

Location of the Tumor

Table 7 shows that the primary tumor was located in the left breast somewhat more often than in the right. This is true within the 3 operation groups and for the total number of patients.

TABLE 6.—Analysis by size of tumor: patients with cancer of the breast in Rockford, Illinois, 1929–54

Size	Type of operation						Total		Relative survival rates*	
	Radical		Modified radical		Simple					
	Number	Percent	Number	Percent	Number	Percent	Number	Percent	5-Year	10-Year
Small	116	49	40	37	48	46	204	45	74(7)	48(10)
Medium	73	31	47	43	32	31	152	34	50(9)	34(11)
Large	29	12	16	15	12	12	57	13	31(14)	19(14)
Unspecified	19	8	5	5	11	11	35	8	60(20)	33(23)
Total	237	100	108	100	103	100	448	100	60(5)	39(6)

*Two standard errors of relative survival rate in parentheses.

TABLE 7.—Analysis by location of tumor (side): patients with cancer of the breast in Rockford, Illinois, 1929–54

Location (side)	Type of operation						Total		Relative survival rates*	
	Radical		Modified radical		Simple					
	Number	Percent	Number	Percent	Number	Percent	Number	Percent	5-Year	10-Year
Right	105	44	52	48	47	46	204	46	56(8)	34(9)
Left	131	55	56	52	55	53	242	54	61(7)	42(9)
Unspecified	1	1	0	0	1	1	2	0	—	—
Total	237	100	108	100	103	100	448	100	60(5)	39(6)

*Two standard errors of relative survival rate in parentheses.

The more frequent left-sided tumors were also associated with higher survival rates than for those on the right side, though the differences in 5- and 10-year relative rates were not statistically significant. The better survival picture for left-sided tumors can perhaps be explained by the fact that they had more favorable size and stage distributions than tumors on the right side (*3*). The respective percentages of small, medium, large, and unspecified size tumors are 47, 33, 11, and 9 for the left side and 43, 36, 15, and 6 for the right side. In regard to the stage distributions, 41, 48, and 11 percent of the left-sided tumors were localized, regional, and indeterminate stage, respectively, in opposition to 33, 57, and 10 percent on the right side.

Table 8 shows the distributions for the 3 operation groups in regard to location of the tumor within the designated areas of the breast. The distributions by type of operation are similar. Also, the distribution of the total number of cases classified as outer, inner, and central correspond well with other breast-cancer series reported in the literature.

As noted previously by Meyer and Smith (*8*), the survival rates were not significantly different, particularly if the data were adjusted for the size and stage distributions among the different breast areas. For the outer, inner, and central areas of the breast, 48, 57, and 28 percent of the tumors were classified as small, respectively, and 39, 51, and 24 percent as localized, respectively.

Thus, the distributions of the side and location of the tumor by type of operation were not significantly different and did not influence survival rates among the 3 operation groups.

Age

Table 9 shows the age distribution of the patients in the 3 operation groups. The patients that underwent simple mastectomy were significantly older as a group than patients who had more radical surgery.

The mean age for all patients during 1929–41 was 54.5; for 1942–47, 58.4; and for 1948–54, 56.7 years. Actually, the mean age of patients with simple mastectomy is highest during the last time period, a fact that is obscured by the small percentage of such operations during 1948–54. When the cases are divided according to stage, the mean ages of patients with localized and regional disease were 55.4 and 56.4 years, respectively, but for indeterminate stage patients, the mean age was 62.6 years. This also reflects the fact that 89 percent of the latter category are simple mastectomies.

Analysis of survival with respect to age is also given in table 9. In conformity with most published data on cancer of the breast, there is little or no relationship between age and survival at 5 years after operation when the relative rate adjusts for the increased mortality from all causes at older age groups. However, at 10 years the relative survival rates appear to be related to age, being increasingly higher at older age groups. However, when data from the larger Connecticut series (*5*)

TABLE 8.—Analysis by location of tumor (area of the breast): patients with cancer of the breast in Rockford, Illinois, 1929–54

Location (area)	Type of operation: Radical Number	Radical Percent	Modified radical Number	Modified radical Percent	Simple Number	Simple Percent	Total Number	Total Percent	Relative survival rates* 5-Year	Relative survival rates* 10-Year
Outer	141	59	62	57	51	49	254	57	59(7)	42(8)
Inner	43	18	16	15	11	11	70	16	67(13)	36(15)
Central	42	18	23	21	25	24	90	20	51(13)	33(14)
Unspecified	11	5	7	7	16	16	34	7	66(20)	37(23)
Total	237	100	108	100	103	100	448	100	60(5)	39(6)

*Two standard errors of the relative rate in parentheses.

TABLE 9.—Analysis by age at operation: patients with cancer of the breast in Rockford, Illinois, 1929–54

Age	Type of operation: Radical Number	Radical Percent	Modified radical Number	Modified radical Percent	Simple Number	Simple Percent	Total Number	Total Percent	Relative survival rates* 5-Year	Relative survival rates* 10-Year
Under 35	12	5	7	6	0	0	19	4	58(24)	32(26)
35–44	46	19	13	12	12	12	71	16	65(12)	33(14)
45–54	64	27	38	35	10	10	112	25	66(10)	40(12)
55–64	54	23	24	22	29	28	107	24	57(11)	45(14)
65–74	49	21	20	19	27	26	96	22	65(14)	54(20)
75 and over	7	3	6	6	20	19	33	7	56(32)	55(59)
Unknown	5	2	0	0	5	5	10	2	—	—
Total	237	100	108	100	103	100	448	100	60(5)	39(6)
Average age	54. 4		55. 1		63. 4		56. 6			

*Two standard errors of relative survival rates in parentheses.

were analyzed for comparison, there was no regular trend in the 10-year survival rates, though there was a poorer prognosis for women under 35 and a more favorable than average prognosis for women 75 years of age and over.

When an adjustment for age was carried out in the survival analysis by time period, type of operation, stage, and size of tumor, no significant changes in the conclusions were elicited regarding the identity of results after simple or radical mastectomy. Thus, the similarity of survival after radical and simple mastectomy is not due to a lower survival after the simple operation that is compensated by a better prognosis among older women.

Miscellaneous Factors

Analysis of the marital status of patients showed a higher proportion of women who had never married among the simple mastectomy group (19%) than among the radical or modified radical groups (10%). There were no consistent or significant differences in survival rates among married and never-married women. Data on the number of children were too incomplete for analysis.

The data were analyzed for the distribution of various descriptive terms for the histologic morphology of the tumors. There was no systematic attempt to grade the tumors. Approximately 75 percent of all tumors were described as adenocarcinoma or scirrhous carcinoma. About 10 percent were described as mucinous, medullary, or papillary. This small group had a slightly higher survival rate than other histologic types; in view of the small number represented, the difference is not statistically significant. Classification of the tumors by histologic type showed no significant relationship with the type of operation.

Data on radiation therapy as part of the primary treatment of breast cancer were unsatisfactory and incomplete. However, records were available to indicate that approximately 25 percent of the patients received some form of postoperative radiation, and, during the earlier period, approximately 5 percent were recorded as receiving preoperative radiation from external sources or interstitial radium. There were no significant differences of distribution of such treatment among the 3 operation groups. The survival rates also were the same among the patients recorded as having received radiation and those for whom no such records were available.

DISCUSSION

Our analysis of the data on surgically treated cancer of the breast in Rockford, Illinois, substantiates the results and the conclusions that have been published by Smith and Meyer (*1*). The survival rates, whether crude or adjusted, were practically identical whether radical or simple

mastectomy was used as the primary treatment. Analyses of a series of possible influencing factors failed to influence this conclusion.

Yet there is no question but that during the 25-year period under study, identifiable differences occurred in the patients, the physicians, and the criteria for the selection of the type of operation. None of the detected differences and evidences of selection could be shown to influence the results to a degree that would alter the main conclusion. However, the factors that were considered were treated perforce as independent variables. It is quite possible, especially since many unknown variables must exist for every one that is identified, that the effect of certain series of variables may be additive or enhancive.

We can only reiterate the comments of Smith and Meyer: "The confusion in our minds relating to the treatment of cancer of the breast has not been resolved by this study . . . we could demonstrate no reason for performing more than simple mastectomy." This confusion is evident in several other comparisons that fail to demonstrate the superiority of the radical operation (*9*). Obviously none of these studies is well controlled, which leaves in doubt the key issue of comparability between patients entering different operation groups.

Additional comparisons between different series of patients will not resolve the basic question of whether the radical mastectomy remains the surgical treatment of choice for breast cancer. The obvious need is for a rigidly controlled clinical trial, with patients entering the procedures to be compared according to a randomization scheme. This need was expressed by the National Advisory Cancer Council at its meeting in February, 1958, when the following resolution was accepted:

"The Council will sponsor, initiate and underwrite national, cooperative, biometrically designed clinical investigations on the comparative effects of radical mastectomy and of simple mastectomy supplemented with radiation for carcinoma of the breast. This resolution carries no implication as to the results of this study, but indicates the belief that it is worth undertaking."

Three full years have elapsed since this resolution was adopted. The re-evaluation of the radical mastectomy and of the simple mastectomy, with or without radiation, remains to be initiated in the United States.

These analyses should not be interpreted as having demonstrated that simple mastectomy is equal or superior to the radical mastectomy for breast cancer. Conversely, it is not demonstrable that the radical mastectomy is superior to the simple mastectomy for breast cancer. The only justified conclusion is that the problem requires an objective, prospective clinical test designed especially for this purpose. One of the requirements for such an investigation is an identical level of surgical proficiency for both procedures to be compared. It should be obvious that the need for and the results of such an investigation do not imply the acceptance of any but the highest standards of total medical care of the patient.

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Intranuclear Inclusions in *Rattus (Mastomys) Natalensis* Infected With Rat Virus [1]

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SUMMARY

Newborn mastomys inoculated with rat virus (RV), an agent originally isolated from tumor-bearing rats, develop an acute fatal disease characterized histopathologically by Feulgen-positive intranuclear inclusions in reticuloendothelial cells in the liver, heart, lungs, kidneys, spleen, and gastrointestinal tract. The inclusions resemble those seen in cells infected with polyoma virus *in vitro*. Although polyoma virus and RV have a number of common biological properties, including the ability of both agents to infect mastomys with involvement of Kupffer cells in the liver, no evidence of proliferative or oncogenic activity has been observed in mastomys inoculated with RV.—J. Nat. Cancer Inst. 27: 1217–1223, 1961.

RAT VIRUS (RV), an agent originally isolated from rats with tumors, grows in rat-embryo cell cultures with production of cytopathic effects and hemagglutinins (*1*). RV is infectious for suckling hamsters, in which it produces either an acute fatal disease (*2*) or a mongoloid type of dwarfism associated with deformities of the teeth (*3*).

Thus far, attempts to infect laboratory rats with RV have been unsuccessful, and neutralizing antibodies have been demonstrated in the serums of all rats tested (*1*). Organotypic cultures of rat submandibular glands have been infected with RV, and intranuclear inclusions have been found in the epithelial and connective-tissue cells of these cultures (*4*). These inclusions were similar to those described in organotypic cultures of mouse salivary gland infected with polyoma virus, and Dawe and his associates have discussed the similarities and differences in biological properties of RV and polyoma virus (*4*).

Rattus (*Mastomys*) *natalensis*, the multimammate mouse, is an African rodent intermediate in size between the rat and the mouse. These animals have been used in microbiological studies because of their high susceptibility to infection by plague bacilli (*5*), *Schistosoma mansoni* (*6*), and

[1] Received for publication July 19, 1961.

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[3] The authors express thanks to Mr. Gebhard Gsell for preparing the photographs, and to Mr. William J. Branigan for technical assistance.

[4] National Institutes of Health, Public Health Service, U.S. Department of Health, Education, and Welfare.

Borrelia duttoni (*7*). They have been of interest to cancer research workers because of the high incidence of spontaneous adenocarcinoma of the stomach reported in a colony of mastomys in Johannesburg (*8*), and because of their susceptibility to tumor induction by polyoma virus (*9*). We were therefore interested in studying the effects of inoculation of RV in mastomys.

MATERIALS AND METHODS

Animals.—Three litters of random-bred newborn mastomys (21 animals) were inoculated with RV when the animals were less than 24 hours old.

Virus.—The virus used was derived from a pool of infected livers and kidneys prepared from the 22d passage of RV in suckling hamsters. This preparation had a titer of 10^7 ID50 on intracerebral inoculation of suckling hamsters.

Methods of inoculation.—Animals were inoculated both subcutaneously (0.05 ml) and intracerebrally in the left parietal region (0.01 ml) by the use of disposable plastic ½-ml syringes and #26 needles.

Autopsies.—Complete autopsies were performed on all animals that were killed or found dead. Tissues were frozen at −20° C for virus isolation or fixed in Zenker-formol for histopathologic studies. Sections were stained routinely with hematoxylin and eosin, and selected sections were prepared with the Feulgen reaction or periodic acid-Schiff reaction, with and without diastase digestion.

RESULTS

Nine of the 21 animals were eaten by the mothers within 2 weeks of inoculation. Four animals were killed when moribund 4, 5, 7, and 9 days after inoculation and complete autopsies were performed. One animal was found dead 30 days after virus inoculation and, although there was some autolysis, gross and microscopic examination of tissues was possible. Seven animals survived more than 1 month with evidence of stunted growth and abnormal dentition. Studies of the dental abnormalities are being conducted by Dr. Paul Baer, of the National Institute of Dental Research, and will be reported subsequently. No tumors have been observed in animals surviving more than a month, although the longest period of observation thus far is 7 months.

Pathology

On gross examination, no significant abnormalities were observed in the animals killed on the 5th, 7th, and 9th days after virus inoculation. The animal killed on the 4th day had marked congestion of the lungs and liver.

Microscopically, in the livers of animals that had been killed when moribund (4th, 5th, 7th, and 9th days after inoculation), intranuclear

purple inclusions were found in Kupffer cells in sections stained with hematoxylin and eosin (figs. 1, 2, and 3). The Kupffer cell nuclei were enlarged with hyperchromatic marginated chromatin, and the masses or clumps of inclusion material were usually surrounded by clear zones or "halos." With high magnification, granules could usually be resolved within the inclusions (figs. 1 and 2). In pyknotic nuclei, the inclusion material was usually in small clumps (fig. 3) and these were often multiple. The inclusions were not periodic acid-Schiff reactive, but were purple in sections prepared with the Feulgen reaction (fig. 4).

Similar inclusions were observed in large mononuclear cells in the epicardium (fig. 5), interstitial tissue of the myocardium, alveolar septa of the lungs, red pulp of the spleen, interstitial tissue of the kidneys, lamina propria and subserosa of the stomach and intestine, and the mesenchyme surrounding developing teeth. The mononuclear cells with inclusions were thought to have been of reticuloendothelial origin, though the possibility that some inclusions were in fibroblasts could not be eliminated. Inclusions could not be identified in epithelial cells, but they were seen in endothelial cells lining small blood vessels in the organs and tissues listed. Large numbers of inclusions were also noted in small mononuclear cells in the external granular layer of the cerebellum (fig. 6) in 2 of the animals (4th and 5th days after virus inoculation).

The inclusions were most numerous in the animals killed on the 4th and 5th days after virus inoculation and less frequent in the animals killed on the 7th and 9th days. No intranuclear inclusions could be definitely identified in the animal that died 1 month after inoculation. There was severe fatty metamorphosis of the liver with focal areas of hepatic necrosis in that animal, but the etiology of this process was not determined.

Uninoculated mastomys 5, 7, and 9 days of age were examined and no intranuclear inclusions were found. Kupffer cells with nuclei filled with dark-purple chromatin were noted; however, the chromatin masses were not surrounded by clear zones and could be distinguished fron the intranuclear inclusions in the animals infected with RV.

Virus Isolations

A suspension of pooled heart, liver, kidney, and spleen was prepared from tissues of the mastomys killed on the 5th day after inoculation. This suspension had a hemagglutinin titer of 1:2560 with the hemagglutination procedure previously described (*2*). It was inoculated intracerebrally into a litter of newborn hamsters and all the 7 inoculated animals were dead within 4 days. A suspension of liver and kidney from the mastomys killed on the 4th day after RV inoculation was also prepared and it had a hemagglutinin titer of greater than 1:128.

DISCUSSION

Although polyoma virus and RV have a number of common biological properties (*4*), including the ability of both agents to infect mastomys,

the response of these animals to the 2 viruses is significantly different. Inoculation of mastomys with polyoma virus has resulted in production of tumors in the kidneys, liver, heart, and subcutaneous tissues (*9*). The mastomys inoculated with RV have developed an acute, rapidly fatal disease with intranuclear inclusions in many organs and tissues, or dwarfism with deformities of the teeth. These findings with polyoma virus and RV in mastomys are similar to results in Syrian hamsters inoculated with the same agents (*2*, *10*).

The presence of Feulgen-positive intranuclear inclusions in mastomys inoculated with RV suggests that RV is a deoxyribonucleic acid (DNA) virus. In this regard, Toolan and her associates found that the agent she isolated from transplantable human tumors, which produces "mongoloid" deformities in hamsters, has properties of a DNA virus (*11*), and one of her virus strains is immunologically similar to RV. Polyoma virus is also a DNA virus, and masses and clumps of Feulgen-positive intranuclear material somewhat similar to those seen in mastomys infected with RV are found in P388 D_1 murine lymphoma cells infected with polyoma virus *in vitro* (*12*).

In mastomys inoculated with polyoma virus, angiomatous tumors of the liver have been observed (*9*), and these apparently are the result of neoplastic transformation of Kupffer cells. In mastomys inoculated with RV, intranuclear inclusions are frequently found in Kupffer cells; however, no evidence of proliferation or neoplastic transformation of these cells has been observed. Further studies of similarities and differences in the biological, chemical, and physical properties of these two rodent viruses may be of importance in the understanding of mechanisms of oncogenesis.

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PLATE 170

FIGURE 1.—Intranuclear inclusion in Kupffer cell of mastomys, 5 days after inoculation of RV. An irregularly shaped collection of granular inclusion material in *center* of nucleus is separated from marginated chromatin by a clear zone. Hematoxylin and eosin. × 1,750

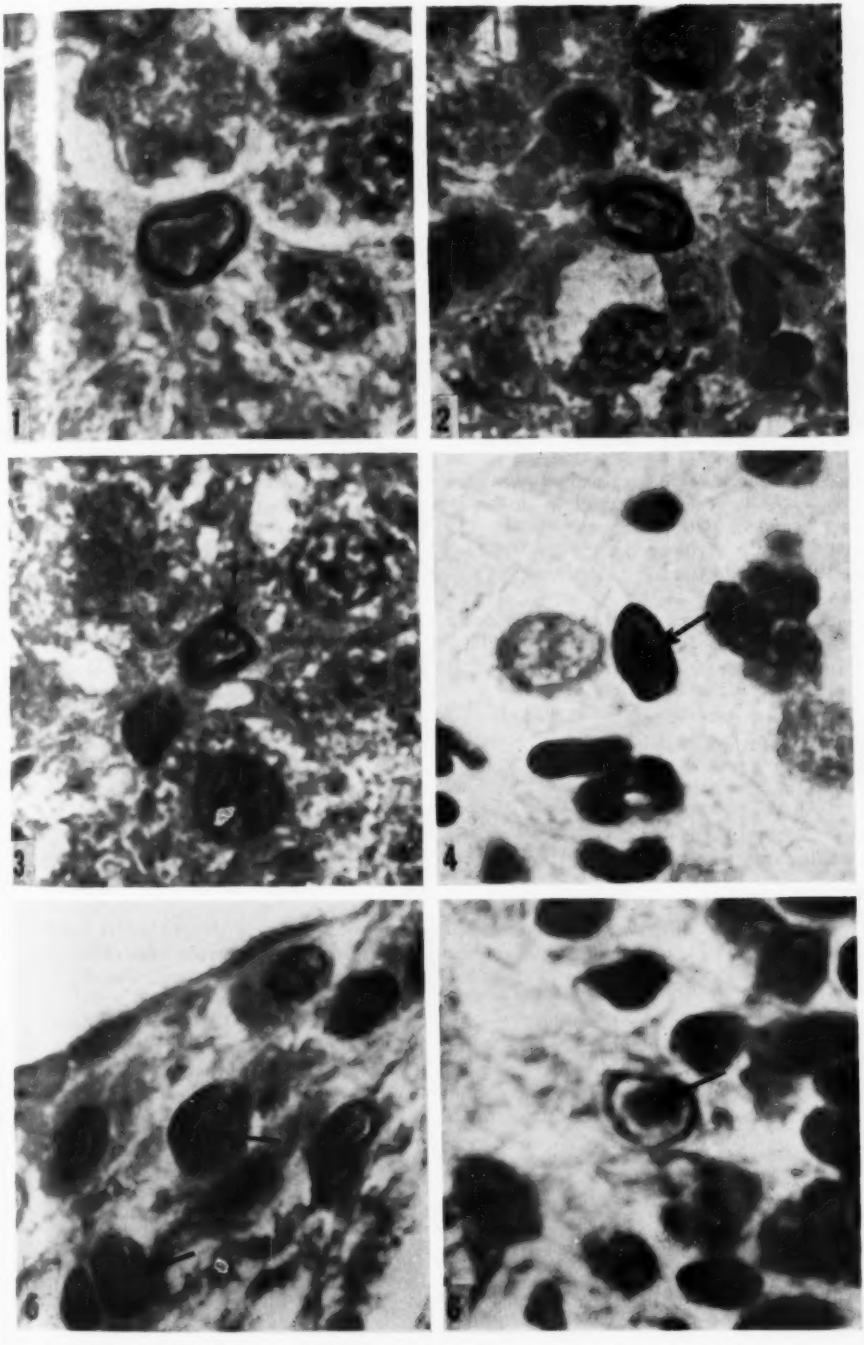
FIGURE 2.—Intranuclear inclusion in Kupffer cell of mastomys, 5 days after inoculation of RV. The inclusion in *center* of nucleus is polygonal and granular. Hematoxylin and eosin. × 1,700

FIGURE 3.—Intranuclear inclusion in Kupffer cell of mastomys, 5 days after inoculation of RV. The nucleus is pyknotic and a small inclusion (*arrow*) is surrounded by a clear zone. Hematoxylin and eosin. × 1,700

FIGURE 4.—Intranuclear Feulgen-reactive inclusion material (*arrow*) in Kupffer cell of mastomys, 5 days after inoculation of RV. Feulgen reaction. × 1,750

FIGURE 5.—Intranuclear inclusions (*arrows*) in mononuclear cells in epicardium of mastomys, 5 days after inoculation of RV. Hematoxylin and eosin. × 1,700

FIGURE 6.—Intranuclear inclusion (*arrow*) in nucleus of cell of external granular layer of cerebellum of mastomys, 4 days after inoculation of RV. Hematoxylin and eosin. × 2,200



Announcements

Sixteenth Annual Symposium on Fundamental Cancer Research

"Conceptual Advances in Immunology and Oncology" will be the subject of the Sixteenth Annual Symposium on Fundamental Cancer Research, to be held March 1, 2, and 3, 1962, at The University of Texas M. D. Anderson Hospital and Tumor Institute. The program will include sessions on "Theories of Antibody Production," "Metabolic Control of Antibody Synthesis," "Genetic Basis of Immune Response," "The Nature of the Antigen-Antibody Reaction," "Transplantation and Immunological Tolerance," and "Cancer Specific Antigens."

Further information may be obtained from the Publications Department, The University of Texas M. D. Anderson Hospital and Tumor Institute, Texas Medical Center, Houston 25, Texas.

Second International Congress of Radiation Research

The Second International Congress of Radiation Research will be held in Harrogate August 6 to 11, 1962, and will be concerned with research into the physical, chemical, and biological effects of ionizing radiations. The Radiation Research Society, in cooperation with the National Academy of Sciences-National Research Council, is exploring possibilities for providing partial support to qualified participants. Applications must be submitted before February 1, 1962. Forms are available from the Committee on Travel Grants, Room 319, 2101 Constitution Avenue, N.W., Washington 25, D.C. Further information about the Congress may be obtained from the Secretary-General, Dr. Alma Howard, Mount Vernon Hospital, Northwood, Middlesex, England.

Manuscripts Approved for JNCI, September 15 to October 15, 1961

"Induction of mammary cancer in agent-free mice bearing pituitary isografts correlated with inherited hormonal mechanisms," by John J. Bittner and Harvey J. Cole.

"Transplantable tumors of the Syrian golden hamsters (*Mesocricetus auratus*)," by Humberto Garcia, Carlo Baroni, and Henry Rappaport.

"The protective effect of parasympatheticomimetic agents on ciliated mucus-secreting epithelium," by H. L. Falk, P. Kotin, and H. M. Tremer.

"Electron microscopy of the H-1 virus: A preliminary report," by Satish Chandra and Helene Wallace Toolan.

"The induction *in vitro* by autonomic drugs of cytoplasmic vacuoles in ascites tumor cells," by Morris Belkin, Walter G. Hardy, Henry C. Orr, and Alan B. Lachman.

"The *p*H of rat tumors and some comparisons with the lissamine green circulation test," by H. Kahler and B. Moore.

"Development of tumors, especially mammary tumors, in agent-free strain DBA/2eB mice," by Margaret K. Deringer.

"Comparative morphologic, histochemical, and serologic studies on the pathogenesis of casein-induced and reticulosarcoma-induced amyloidosis in mice," by H. E. Christensen and R. Rask-Nielsen.

"Virus infection of cells in mitosis. III. Cytology of mitotic and amitotic HEp-2 cells infected with measles virus," by Bernard Roizman and Ann E. Schluederberg.

"Pathogenesis of virus-induced Rous sarcoma. I. Distribution of virus and tumor foci in chicks and turkeys," by Herbert J. Spencer and Vincent Groupé.

"Pathogenesis of virus-induced Rous sarcoma. II. Dynamics of tumor development and viral growth patterns in chicks," by Herbert J. Spencer and Vincent Groupé.

"Birth characteristics of children dying of malignant neoplasms," by Brian MacMahon and Vaun A. Newill.

"Virus of avian myeloblastosis. XIX. Protein, polysaccharide, lipide, and nucleic acid of myeloblasts and cytidine uptake *in vitro*," by J. R. Sommer, David Weinstein, Caroline Becker, G. S. Beaudreau, Dorothy Beard, and J. W. Beard.

U.S. GOVERNMENT PRINTING OFFICE: 1961